"ANALYSIS OF ORANGE COMPLEX BACTERIA IN THE MICROBIOME OF WHOLE SALIVA IN PERIODONTAL HEALTH AND PERIODONTITIS INDIVIDUALS USING NEXT GENERATION SEQUENCING TECHNOLOGY"

Dissertation submitted to

THE TAMILNADU Dr. M.G.R. MEDICAL UNIVERSITY

In partial fulfillment for the Degree of

MASTER OF DENTAL SURGERY

BRANCH II

PERIODONTOLOGY

MAY 2019

THE TAMILNADU Dr. M.G.R MEDICAL UNIVERSITY
CHENNAI

DECLARATION BY THE CANDIDATE

I hereby declare that this dissertation titled **"ANALYSIS OF ORANGE COMPLEX BACTERIA IN THE MICROBIOME OF WHOLE SALIVA IN PERIODONTAL HEALTH AND PERIODONTITIS INDIVIDUALS USING NEXT GENERATION SEQUENCING TECHNOLOGY"** is a bonafide and genuine research work carried out by me under the guidance of **Dr. K.V.ARUN, M.D.S.**, Professor and HOD, Department of Periodontology, Ragas Dental College and Hospital, Chennai.

Dr. ENNET CYNTHIA JOHNS
Post Graduate Student,
Department of Periodontology,
Ragas Dental College & Hospital,
Chennai.

Date: 31-1-19

Place: Chennai

CERTIFICATE

This is to certify that this dissertation titled **"ANALYSIS OF ORANGE COMPLEX BACTERIA IN THE MICROBIOME OF WHOLE SALIVA IN PERIODONTAL HEALTH AND PERIODONTITIS INDIVIDUALS USING NEXT GENERATION SEQUENCING TECHNOLOGY"** is a bonafide record of work done by **Dr. ENNET CYNTHIA JOHNS** under my guidance during the study period 2016-2019.

This dissertation is submitted to **THE TAMILNADU Dr. MGR MEDICAL UNIVERSITY** in partial fulfilment for the degree of **MASTER OF DENTAL SURGERY, BRANCH II- PERIODONTOLOGY.** It has not been submitted (partial or full) for the award of any other degree or diploma.

Guide and Head of the Department:

RAGAS DENTAL COLLEGE Chennai

Dr. K. V. Arun, M.D.S.,

Professor and Head of the Department

Ragas Dental College & Hospital,

Chennai.

Dr.K.V.ARUN MDS
Head of the Department
Department of Periodontics
Ragas Dental College and Hospital
Chennai - 600 119.

Dr. N.S.Azhagarasan, M.D.S.,

Principal,

Ragas Dental College &Hospital

Chennai.

PRINCIPAL
RAGAS DENTAL COLLEGE AND HOSPITAL
UTHANDI, CHENNAI-600 119.

Department of Periodontics,
Ragas Dental College & Hospital,
#2/102, ECR, Chennai-119

THE TAMILNADU Dr. MGR MEDICAL UNIVERSITY
CHENNAI

PLAGIARISM CERTIFICATE

This is to certify that this dissertation work titled "**ANALYSIS OF ORANGE COMPLEX BACTERIA IN THE MICROBIOME OF WHOLE SALIVA IN PERIODONTAL HEALTH AND PERIODONTITIS INDIVIDUALS USING NEXT GENERATION SEQUENCING TECHNOLOGY**" of the candidate **DR. ENNET CYTHNIA JOHNS** for the award of **MASTER OF DENTAL SURGERY** in the branch of **PERIODONTOLOGY**.

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Date: 31-1-19
Chennai.

Guide & Supervisor sign with Seal.

Dr. K.V. Arun, M.D.S.,
Professor and head of the department
Department of Periodontology,
Ragas Dental College & Hospital
Chennai.

Department of Periodontics,
Ragas Dental College & Hospital
2/102, ECR, Chennai-119

Acknowledgement

ACKNOWLEDGEMENT

First and foremost, I would like to thank **ALMIGHTY LORD** for bestowing his abundant grace and blessings upon me, without which pursuing this degree would not be possible and then **My Dad, Dr. PREM KUMAR JOHNS** for his constant prayers and blessings from above, which has lead me to take this path.

I take this opportunity to thank **Dr. N.S. Azhagarasan, MDS**, Principal, Ragas Dental College and Hospital for his guidance during my postgraduate course at Ragas Dental College and Hospital.

I express my sincere thanks to my most respected and beloved Professor **Dr.K.V.Arun,MDS,** professor and head of the department of Periodontics, Ragas dental college Chennai, for his valuable advice, guidance, patience and insight throughout my post graduate course. Truly blessed and feeling grateful to have him as my mentor and guide.

I would like to express my gratitude to **Dr. G. Sivaram,MDS,** Professor who has constantly inspired me and has unceasingly guided me throughout my study process with his constant support**.**

I would like to thank **Dr.Shivakumar**, **MDS**, **Dr. Ramya Arun, MDS,** Professor who has generously guided with the valuable advice and constant encouragement which helped me throughout the post-graduation course.

I would like to express my profound sense of gratitude to **Dr. Swarna Alamelu, MDS,** Reader and **Dr. Archana Meenakshi, MDS,**Reader for their constant support and encouragement throughout my tenure.

I would like to thank **Dr. Deepavalli, MDS,** Senior Lecturer and **Dr. A.R. Akbar, MDS,** Senior Lecturer for their continuous support and guidance. I would also like to thank **Dr. R.S. Pavithra, MDS,** Senior Lecturer, **Dr. J. Velkumar, MDS,** Senior Lecturer and **Dr. Sreemohana, MDS,** Senior Lecturer for their constant support.

I remain ever grateful to my batch mates **Dr.Ali firouzi, Dr.Asha, Dr.Kavipriya, Dr. Krupa** and **Dr. Santosh kumar,** for their constant support and encouragement. I thank all my seniors, especially **Dr.Guhanathan, Dr.Arvinth Vishnu, Dr.Latha** and **Dr.Anisha**, and my juniors, especially **Dr.Indhumathi** who has always been beside me throughout this process and also **Dr.Raghamalika, Dr. Aswathy, Dr. Davis and Dr.Meenakshi** for their timely help, support and encouragement. I would also like to thank my juniors **Dr.Gayathri, Dr.Laxmi Prasanna, Dr. Shunmugapriya** and **Dr.Shalini.**

I would like to thank my friends **Dr.Chinnu, Dr.Darlene, Dr.Amruthasree** and **Dr.Sheril Selvan** who has always been beside me through this journey

I extend my gratitude to **Mr. Jotheeswaran, Mrs. Parvathi, Mrs. Rosamma, Mr. Chellapan, Mrs. Mala** and **Ms. Sheela** for their timely help during the tenure.

I would like to thank **Dr. Darshan** for helping me with the statistical part of this dissertation.

I would like to express my heartfelt gratitude and love to my mom**,** **Mrs. Hazel Johns** for her sacrifices and also for nurturing and supporting my vision with unceasing faith and hope in me without whom I wouldn't have pursued this degree.

I would like to express my heartfelt gratitude and love to **Dr.Johnson** for believing in me**,** the one who not only envisioned my dad's dream but also has been my dose of inspiration and pillar of support in pursuing this degree.

I would love to thank my family **Dr.Ticksy Johns, Prarthanaa, Prarthvikh, Mr.Freddy** who have always been a constant support in various ways in achieving my dreams.

I would like to extend my love and gratitude to my people **Dr.Rinu** and **Dr.simi** for always believing in me and who has been a biggest support throughout this study process.

LIST OF ABRREVIATIONS

PSD	**POLYMICROBIAL SYNERGY AND DYSBIOSIS**
DNA	**DEOXYRIBONUCLEIC ACID**
rRNA	**RIBOSOMAL RIBONUCLEIC ACID**
HOMIM	**HUMAN ORAL MICROBIOME IDENTIFICATION MICROARRAY**
NGS	**NEXT GENERATION SEQUENCING**
MSR	**MISEQ REPORTER SOFTWARE**
HOMD	**HUMAN ORAL MICROBIOME DATABASE**
OTU	**OPERATIONAL TAXONOMIC UNIT**
PCR	**POLYMERASE CHAIN REACTION**

SOLID	**SUPPORTED OLIGONUCLEOTIDE LIGATION AND DETECTION**
BLAST	**BASIC LOCAL ALIGNMENT SEARCH TOOL**
HOT	**HUMAN ORAL TAXON NUMBER**
MiSeq	**METAGENOMIC SEQUENCING**
RSB	**RESUSPENSION BUFFER**

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Introduction

INTRODUCTION

The majority of the cells in the human body are of microbial and not mammalian origin.[112] These microorganisms form the resident microflora described as the microbiome that is unique to the individual and the ecological habitat. The oral microbiome has been divided into distinct ecological niches in the tongue, buccal mucosa and the floor of the mouth.[55] In the periodontal environment there are two distinct microbiomes in the supra gingival and subgingival plaque that differ in characteristics and ability to initiate disease process.

Gingivitis is a reversible non-specific inflammatory reaction of the marginal gingiva to plaque accumulation, whereas Periodontitis is a non-resolving and irreversible condition resulting in loss of attachment apparatus potentially leading to tooth loss. [126]

Existing evidence indicates that gingivitis precedes the onset of periodontitis; However, not all gingivitis cases develop into periodontitis. [67] The reason for this is that accumulation of plaque bacteria is necessary but not sufficient by itself for the development of periodontitis.[73,94]Individual disease susceptibility plays a major role in the progression from gingivitis to periodontitis. This individual susceptibility has been examined extensively and a host of genetic, inflammatory, host tissue and microbial biomarkers have been developed to assess periodontal disease progression.[107]

Over the past 50 years, understanding and characterization of dental plaque has undergone significant evolution from Nonspecific plaque hypothesis and Specific plaque hypothesis to Ecological plaque hypothesis. Currently, pathogenesis of periodontal disease is explained by "Polymicrobial Synergy AND Dysbiosis Model (PSD)" proposed by **HAJISHENGALIS ET AL.**[38] This model states that the dysbiotic environment and polymicrobial synergy are the key events that lead to development of periodontitis rather than individual bacterial species.

Dysbiosis is a symbiotic relationship that has changed due to decrease in number of beneficial symbionts and/or an increase in number of pathobionts.[7] Research over the past decade has led to the recognition of these dysbiotic microbiomes residing in the various oral ecological niches including mucosal surfaces and saliva. [141]

Saliva is a body fluid essential for the maintenance of health of the oral cavity including the periodontium. Whole saliva is a complex mixture of oral fluids including secretions of the major and minor salivary glands; constituents of non-salivary origin derived from GCF, desquamated epithelial cells and food debris. It plays an important role in maintaining the homeostasis of the periodontal tissues through the anti-bacterial effects exerted by the Immunoglobulins and other antimicrobial peptides present in it.

Although saliva does not have a resident microflora, it has been postulated that salivary microorganisms may play an important role in the

etiology and propagation of periodontal diseases.[80] Bacterial translocation through saliva has been proposed to play a role in transfer of subgingival bacteria from uninfected to infected sites and recolonization of treated sites in the periodontium. [103]

Subgingival microbiota has been divided into complexes, based on their association with health and various disease severities. The yellow, green and purple complexes being early colonizers that favour colonization of orange and red complexes that have been associated with periodontal disease activity.[121,120]

Although the PSD model proposes a role for the microbiome as a whole in the etiopathogenesis of periodontal disease, **Hajishengalis** has postulated that the red complex bacteria may act as keystone pathogens, while pathobionts including bacteria belonging to the orange complex help in exaggerating the inflammatory response.

The **orange** complex is constituted by *Fusobacterium nucleatum, Prevotella intermedia, Prevotella nigrescens, Peptostreptococcus micros, Streptococcus constellatus, Eubacterium nodatum, Campylobacter showae*, *Campylobacter gracilis, Campylobacter rectus.* There is voluminous literature on the relative abundance and virulence of these organisms in subgingival plaque. However, their presence and role in saliva is yet to be fully characterized, especially in Indian populations.

Current trend in sequencing of microbiome is based on next generation sequencing. Next-generation sequencing (NGS) is a type of DNA sequencing technology that uses parallel sequencing of multiple small fragments of DNA to determine genetic sequences.[105] In contrast to Sanger sequencing, the speed of sequencing and amounts of DNA sequence data generated with NGS is exponentially greater and are produced at significantly reduced costs.[6]

This study proposes to evaluate the orange complex species in the saliva of individuals in periodontal health and disease using next generation sequencing so as to ascertain the possibility of using them as microbial risk markers of disease activity.

Aim and Objectives

AIM AND OBJECTIVES

AIM:

- To evaluate the Orange complex species in the saliva in periodontal health and periodontitis patients using Next Generation Sequencing technique with Illumina sequencing method.

OBJECTIVES:

- To evaluate the Orange complex species in the saliva in periodontal health and periodontitis patients using Next Generation Sequencing technique with Illumina sequencing method.
- To compare the frequency distribution of salivary Orange complex bacteria in periodontitis with that of periodontally healthy individuals.

Review of Literature

REVIEW OF LITERATURE

The human body is composed of more than 10^{14} cells, out of which only 10% are mammalian.[116] Most of these cells are the microorganisms that make up the resident microflora found on all environmentally exposed surfaces of the body, and the human "microbiome" is reported to have a metabolic capacity equivalent to that of the human liver.[87,89] It is estimated that between 300 and 400 different species are capable of colonizing the mouth, and any individual may typically harbor 150 to 200 different species. Counts in subgingival sites range from about $l0^3$ in healthy, shallow sulci to more than 10^8 in deep periodontal pockets.[120,121,122] The microflora of the mouth is distinctive because of the characteristic biological and physical properties of each site despite the potential movement of microorganisms between sites. The tooth provides a surface for the colonization of a diverse array of bacterial species. Bacteria may attach to the tooth itself, to the epithelial surfaces of the gingiva or periodontal pocket, to underlying connective tissues, if exposed, and to other bacteria attached to these surfaces. These observations illustrate a key concept that the properties of the habitat are selective and dictate which organisms are able to colonize, grow and be minor or major members of the community.[87,89.]

The human mouth harbors one of the most diverse microbiomes in the human body, including viruses, fungi, protozoa, archaea and bacteria. The microorganisms in the human oral cavity is also referred to as the oral

microflora, oral microbiota, or more recently as the oral microbiome. The term microbiome was coined by **Joshua Lederberg**[63] "to signify the ecological community of commensal, symbiotic, and pathogenic microorganisms that share our body space and have been all but ignored as determinants of health and disease". The human microbiome can be classified into two distinct types: a core microbiome and a variable microbiome.[132] The core microbiome is shared among all individuals and is comprised of the predominant species that exist under healthy conditions at different sites of the body.[132,49,51,123] The variable microbiome is exclusive to the individual and has evolved in response to unique lifestyle, and phenotypic and genotypic determinants. Although individuals share microbiota at similar sites of the body, there are varying differences at the species and strain level of the microbiome that can be as inimitable to the individual as is the fingerprint.[23]

The oral cavity is highly populated with numerous polymicrobial communities, each occupying highly specific niches that differ in both anatomic location and nutrient availability. Oral host colonization is a reflection of the proficiency of bacteria to adapt to a variety of different niches through high rates of genetic recombination.[106]

Dysbiosis is defined as change/perturbations in the structure and composition of resident commensal bacterial communities relative to the community found in healthy individuals. Dysbiosis is a symbiotic relationship which has gone astray caused by a decrease in the number of beneficial symbionts and/or an increase in the number of pathobionts. Biological

properties that help to maintain stability in the microbiome are important for sustaining symbiotic environment and for prevention of dysbiotic state.

Dental plaque-associated periodontitis is classified into two main categories – chronic and aggressive, with the latter being characterized by increased severity and rate of progression and generally earlier onset.[4] These general descriptions are likely to encompass a spectrum of related diseases, however, which are clinically difficult to differentiate. A specific microbiota is associated with advanced periodontitis, although whether certain species are responsible for the initiation of the disease or the inflammation and pocket formation create conditions that periodontitis-associated bacteria prefer remains unclear.[74] Host susceptibility is clearly of primary importance and tobacco smoking is a strong environmental factor.[134]

Paster et al[15,] stated that there exist 700 bacterial species in oral cavity. Periodontal disease condition becomes nutritionally richer environment for bacteria, thus higher bacterial diversity can be identified under diseased conditions but there may be interindividual differences in the disease group which cannot be ruled out**.** Gingivitis or Periodontitis is accompanied by a shift in the oral bacterial community, structure and composition.[97,98.]

The microbial etiology of periodontitis has been extensively researched for the past few decades, and it is now well known that periodontitis is not associated with a single micro-organism but is a consortium of bacteria participating in the initiation and progression of the disease process.[110] The

most significant consequence of biofilm formation on the tooth surface is the continuous release of bacterial toxins into the local microenvironment. The composition of the subgingival microbial flora and the level of pathogenic species differ from each individual as well as from site to site. Dental plaque has been defined as the microbial community that develops on the tooth surface, embedded in a matrix of polymers of bacterial and salivary origin. Dental plaque is formed via an ordered sequence of events, resulting in a structurally and functionally organized species-rich microbial biofilm.[11,83,84.]

LOE'S experimental gingivitis recognized the etiologic role of plaque in periodontal disease and firmly established that it was involved in the initiation and progression of periodontal diseases[73]. It has been generally accepted that periodontal diseases appear to be initiated by a relatively limited number of periodontal pathogens in the complex dental biofilm, and they represent a small part of the approximately 600 bacterial species that have been found to colonize dental surfaces over and below the gingival margin and oral mucous membranes.[101] Clinical and experimental evidences in the last few decades confirm that certain bacterial strains in the periodontal environment can induce gingival tissue inflammation and bone destruction. These bacterial strains are defined as periodontal pathogens.[98] Only a small percentage of the dental biofilm bacteria are defined as pathogenic for the periodontal tissues and these bacteria, even when present in very small quantities, possess the ability to damage the periodontal structures.

According to the criteria proposed by **Socransky and Haffajee**[121,122], a periodontal microorganism has to meet certain conditions to be considered a potential pathogen: to be associated with the disease by means of increased number in diseased patients and sites; to be reduced or eliminated after treatment and, with the healing, to be capable of provoking the destructive host response; to possess the capacity to cause the disease in experimental animal models; to demonstrate production of virulence factors known to cause periodontal destruction.[121,122] The ideas about how changes in dental plaque relate to a shift from oral health to disease have changed over time. Over the past 50 years, the understanding and characterization of dental plaque have undergone significant evolution.

Non-specific plaque hypothesis

The Non-specific plaque hypothesis was based on work of researchers **Black (1884)[8] and Miller (1890)[14]**. This hypothesis stated that periodontal disease is due to bacterial accumulation, irrespective of its composition. This implies that no one specific bacterial species is any more significant than another in its ability to cause periodontal disease.[40] Any accumulation of microorganisms at or below the gingival margin would produce irritants, leading to inflammation. The inflammation, in turn, was responsible for the periodontal tissue destruction.[120] It was proposed that the entire microbial community of plaque that accumulated on tooth surfaces and in the gingival crevice contributed to the development of periodontal disease through the production of virulence factors and noxious products that initiated

inflammation, challenged the host defense system, and resulted in the destruction of periodontal tissues. Thus, increases in the amount of plaque (quantity), as opposed to specific pathogenic microorganisms (quality) found in the plaque, were viewed as being primarily responsible for inducing disease and disease progression.[35] It appears that different combinations of indigenous bacteria, rather than just a single species, can produce the pathogenic potential necessary to cause progression from gingivitis to destructive periodontitis.[128]

Theilade and Attstorm (1985)[128] observed inflammation and loss of hemidesmosomes between the junctional epithelium and the teeth in dogs are seen ahead of the bacteria. Although the amount of plaque present may correlate well with disease severity in cross-sectional studies, it correlates poorly in longitudinal studies.

Non-specific plaque hypothesis is valid for the development of gingivitis but not for the development of periodontitis, which is a multifactorial disease.[94] This concept also failed to explain why all gingivitis not progress to periodontitis and why some individuals with increased plaque showed little overt periodontitis and some individuals with very little plaque manifested with aggressive and advanced forms of periodontitis.[120,121,122] This hypothesis does not consider variations in the dental biofilm that may affect its pathogenicity or, most importantly, host determinants.

Specific plaque hypothesis

According to this hypothesis proposed by Walter J. Loesche, periodontal disease is the result of an infection with a single specific pathogen.

Studies on the microbial etiology of various forms of periodontitis support the specific plaque hypothesis, which proposes that only certain microorganisms within the plaque complex are pathogenic. For example, *Aggregatibactor actinomycetacomitans* was identified as a specific pathogen in localized aggressive periodontitis.[90] Despite the presence of hundreds of species of microorganisms in periodontal pockets, fewer than 20 are routinely found in increased proportions at periodontally diseased sites. These specific virulent bacterial species activate the host's immune and inflammatory responses that then cause bone and soft tissue destruction.

Socransky and colleagues[120,121,122] in 1998 recognized that early plaque consists predominantly of gram-positive organisms and that if the plaque is left undisturbed it undergoes a process of maturation resulting in a more complex and predominantly gram-negative flora. These investigators assigned the organisms of the subgingival microbiota into groups, or complexes, based on their association with health and various disease severities.[35] The yellow, green and purple complexes were the early colonizers that favor the colonization of orange and red complexes. The red complex bacteria included Bacteroides forsythus (now *Tannerella forsythia),* *Porphyromonas gingivalis* and *Treponema denticola* and they were significantly associated with periodontitis. This hypothesis failed to explain why the putative periodontal pathogens like *Porphyromonas gingivalis, Tannerella forsythia* are frequently found in healthy periodontal sites.

Ecological plaque hypothesis

Ecological plaque hypothesis was proposed by **Philip D. Marsh**[83,84] in 1994. It was proposed to describe and explain the dynamic relationship between the resident microflora and the host in health and disease in ecological terms. The theory underpinning this hypothesis in the context of periodontal disease is that changes in the environment, increase the competitiveness of the putative pathogens (which, if present in health, are generally only at low and clinically insignificant levels) at the expense of species associated with oral health and upregulate the expression of virulence factors. Importantly, there is acknowledgement of a clear link between local environmental conditions and the activity and composition of the biofilm community. Any change to the environment induces a response in the microflora, and vice versa.[83,84]

Keystone Pathogen Hypothesis

The Keystone Pathogen Hypothesis indicates that certain low-abundance microbial pathogens can cause inflammatory disease by increasing the quantity of the normal microbiota and by changing its composition.[38,39] When disease develops and advanced stages are reached, the keystone pathogens are detected in higher numbers.[120,121,122]

Polymicrobial Synergy and Dysbiosis Model

PSD model of pathogenesis states that periodontitis is initiated by a broadly based dysbiotic, synergistic microbiota as against the traditional view that it is caused by a single or several periopathogens like red complex

bacteria.[38,39] This dysbiotic, synergistic microbiota environment retards the host-microbe homeostasis and offers its transition to a chronic inflammatory state. It is the interaction between the subgingival community of microorganisms and local immune responses that ultimately leads to bone and connective tissue attachment loss.[38,95,103.] **(Figure -1)**

In the periodontal ecosystem wide variety of bacteria (or specific combinations of genes within the community) are present, which might be able to fulfil distinct roles that converge to form and stabilize a disease-provoking microbiota. Hence, there will be a number of core requirements for a potentially pathogenic community to arise. (i) Bacterial constituents will express the relevant adhesins and receptors to allow assembly of a heterotypic community. (ii) Individual members of the community will be physiologically compatible or at least non-antagonistic. (iii) The combined activities of the community will resist the host innate and acquired immune responses and contribute to tissue inflammation through, that is by proteolytic activity and cytokine induction.

Dental plaque obtained from healthy sites shares the capacity of disease-associated plaque to induce strong inflammatory responses through Toll-like receptor activation.[143] However, for pathogenic potential to be realized, the activities of a keystone species such as *P. gingivalis* are required. These organisms engage in two-way communication with the community inhabitants, in particular the accessory pathogens, to both disrupt host immune surveillance and increase the pathogenicity of the entire group. This more

specialized dysbiotic role will be precise to fewer organisms. The identification of keystone and accessory pathogens from the catalogue of organisms elicited by microbiome projects will present the next major challenge in periodontal disease research and, perhaps, in other inflammatory diseases with a complex polymicrobial etiology.[38,39] Moreover, scientific researches towards the PSD model would help to pursue the therapeutic approaches for periodontitis.

Mixed microbial communities provide opportunities for competitive and co-operative interspecies interactions, and such interactions shape the nature and function of the entire assemblage.[45,85,98.]

SUBGINGIVAL MICROBIOME

Members of the human oral microbiome were the first bacteria ever to be observed. In 1683, **Antonie van Leeuwenhoek** used his microscope to observe a large number of what he named "animalcules" in scrapings taken from his teeth. Over 200 years later, the seminal work of **Koch, Pasteur** and their contemporaries identified the animalcules as microorganisms and the first isolates of cultivable members of the oral microbiome which were later exquisitely studied in the laboratory.

The subgingival microbiome is the community of microorganisms inhabiting the subgingival environment. The microbial composition of subgingival plaque at periodontally diseased sites has been extensively studied.[120,121,122,29] In a landmark study by **Socransky** and **Haffajee** in 1998, they attempted to define bacterial communities existing as different complexes

in subgingival plaque by studying 13,261 plaque samples from 185 subjects using whole genomic DNA probes and checkerboard DNA-DNA hybridization. They defined 5 major bacterial complexes identified by different clustering and ordination techniques. Red complex which exclusively comprised of *Tanerella forsythia, Porphyromonas gingivalis and Treponema denticola*,[120] were thought to be mostly associated with Periodontitis and to clinical measures like probing depth and bleeding on probing. (**Figure-3)**

A series of culture studies of subgingival plaque taken from subjects with different forms of periodontal disease and health reported a shift in the subgingival microbiota as the periodontium progressed from health through gingivitis to periodontitis.[88,55] **Liu et al and Cheng et al** investigated bacterial diversity between periodontal health and disease status using 16S rRNA amplicon sequencing and showed that there is a shift in the composition of the oral microbiota between healthy and diseased samples[72].

Species associated with periodontitis include *Porphyromonas gingivalis, Treponema denticola and Tannerella forsythia* which were originally associated with the disease in culture-based studies and subsequently confirmed with whole-genomic DNA probes. Culture-independent studies have expanded the range of disease-associated organisms to include Anaeroglobus geminatus, Eubacterium saphenum, Filifactor alocis, Porphyromonas endodontalis, Prevotella denticola and unnamed Bacteroidetes and Fretibacterium phylotypes.[58,74].

Microbiome Studies in Periodontal Disease:

Chronic periodontitis:

Kroes et al[60] conducted a study using 16S rRNA gene sequencing and Sanger sequencing in order to evaluate the subgingival microbiome in chronic periodontitis and founded that there were 77 phylotypes involved in the disease out of which 48% were novel.

Paster et al[15,97,98] did a study using Sanger technique under different periodontal conditions and founded that there was a total of 347 phylotypes involved in the disease and among them 215 were novel.

Kumar et al[61,138] found that 274 phylotypes were associated in the chronic periodontitis while evaluating the subgingival microbiome using Sanger sequencing in health and periodontitis. Phylotypes associated with periodontitis were identified as Peptostreptococcus spp., Filifactoralocis, Megasphaera sp., Desulfobulbus sp., Dialister spp. Campylobacter spp., Selenomonas sp., Deferribacteres sp., Catonella sp., Tannerella forsythia, Streptococcus spp., Atopobium sp., Eubacterium sp. and Treponema sp. Phylotypes associated with health were Veillonella sp., Campylobacter gracilis, Campylobacter showae, Abiotrophiaadiacens, Eubacteriumsaburreum, Gemella sp., Streptococcus sanguis, Streptococcus mutans, Capnocytophagagingivalis, Rothiadentocariosa, Eubacterium sp. And Selenomonas sp.

Griffen et al[32] founded 16 phyla, 106 genera and 596 species in chronic periodontitis using high throughput sequencing. He also stated that

health associated species may also be present in the disease but in low abundance.

Abusleme et al[1] did a study in 22 subjects to understand the ecology of oral subgingival communities in health and periodontitis and elucidated the relationship between inflammation and the subgingival microbiome using 454-pyrosequencing of 16S rRNA gene libraries and quantitative PCR. He found that periodontitis communities had higher proportions of Spirochetes, Synergistetes, Firmicutes and Chloroflexi, among other taxa, while the proportions of Actinobacteria, particularly Actinomyces, were higher in health. Total Actinomyces load, however, remained constant from health to periodontitis.

Hong et al[2,108] founded that two variant of microbiome profiles can be seen in periodontitis which includes clusters A and cluster B which was derived clustering analyses of microbial abundance profiles. In subjects with cluster A communities there was an increased proportion of different periodontitis-associated species, health-associated species and core taxa which is prevalent both in health and periodontitis. Cluster B communities showed increased proportions of certain periodontitis-associated organisms, such as Porphyromonas gingivalis, Tannerella forsythia and Treponema denticola, and taxa recently linked to periodontitis. The cluster B community showed a positive correlation with periodontitis extent.

Chi-Ying Tsai[131] did a study in Taiwanese individuals with chronic periodontitis with 16S rRNA metagenomic approach. He determined the

subgingival microbiota and demonstrated a microbial shift from health to disease.

Camelo-Castillo[12,41] evaluated the relationship between the chronic periodontitis-associated subgingival microbiota and clinical inflammation. Subgingival bacterial samples from periodontal patients were studied by pyrosequencing PCR products of the 16S rRNA gene and by real-time PCR. He founded that 16S pyrosequencing revealed that increased inflammation, at sites with periodontitis, is associated with a more diverse subgingival microbiota and specific changes in the bacterial composition, involving "established" periopathogens, symbionts and novel low-abundance pathobionts.

Gingivitis:

Kistler et al [53,100] in 40 patients studied using 454 pyrosequencing of the 16S rRNA genes and bacterial culture, to characterize the composition of plaque during the transition from periodontal health to gingivitis and found that species-level phylotypes positively and negatively correlated with gingivitis. Increased community diversity and significant shifts in microbiome structure after two weeks of oral hygiene abstention was reported. All of the healthy volunteers developed gingivitis after two weeks. Pyrosequencing yielded a final total of 344 sequences after filtering, with a mean length of 354 bases, that were clustered into an average of 299 species-level Operational Taxonomic Units (OTUs) per sample. Changes in the relative abundance of OTUs during the transition from health to gingivitis were correlated to

bleeding on probing (BOP) scores and resulted in the identification of new health and gingivitis associated taxa. Comparison of the healthy volunteers to the periodontitis patients also confirmed the association of a number of putative periodontal pathogens with chronic periodontitis. Taxa associated with gingivitis included Fusobacterium nucleatum subsp. polymorphum, Lachnospiraceae sp., Lautropia sp. and Prevotellaoulorum, whilst Rothiadentocariosa was associated with periodontal health.

Abusleme et al[1,30,51] conducted a study in 2013 to analyze the ecology of oral subgingival communities in health and periodontitis and elucidate the relationship between inflammation and subgingival microbiome. They characterized the subgingival microbiome of 22 subjects with chronic periodontitis, and compared it with those from 10 healthy subjects. They found a higher biodiversity and biomass in periodontitis compared to health, with periodontitis having higher proportions of Spirochetes, Synergistetes, Firmicutes and Chloroflexi; while the proportions of Actinobacteria like Actinomyces were increased in health. They also showed an association between biomass and community structure in periodontitis with proportions of specific taxa correlating with bacterial load.

In a study by **Yan Li et al in 2014**[22] they examined the phylogenetic and functional gene differences between 25 periodontal and 12 healthy subjects using MiSeq sequencing. 39 genera were significantly different between healthy and periodontitis group and Fusobacterium, Porphyromonas, Treponema, Filifactor, Eubacterium, Tannerella, Parvimonas,

Peptostreptococcus and Catonella showed higher relative abundances in the periodontitis groups. A variety of genes involved in virulence factors, amino acid metabolism and glycosaminoglycan and pyrimidine degradation were enriched in periodontitis whereas genes involved in amino acid synthesis and pyrimidine synthesis exhibited a significantly lower relative abundance compared with healthy group.

Hong BY et al[2,45] conducted a study in 2015 to explore the existence of different community types in periodontitis and their relationship with host demographic, medical and disease-related clinical characteristics. Their results suggested 2 types of communities (A and B) which existed in the 34 subjects with periodontitis. Type B communities harbored greater proportions of certain periodontitis associated taxa like *Porphyromonas gingivalis, Tannerella forsythia and Treponema denticola* and other recently linked periodontitis associated ones. In contrast, type A communities had increased proportions of different species and were also enriched for health associated species and core taxa.

Hui Zheng et al in (2015)[146] performed a study to analyze the microbial characteristics of oral plaque from peri-implant pockets of 10 healthy implants, 8 peri-implant mucositis sites and 6 peri-implantitis sites using pyrosequencing of 16S rRNA gene, and reported an increase in microbial diversity in subgingival sites of ailing implants compared with healthy implants. Periodontal pathogens like *Porphyromonas gingivalis, Tannerella*

forsythia and Prevotella intermedia were clustered into modules in the peri implant mucositis network.

Baochen Shi et al[117] in 2015 aimed to determine the dynamic changes in the subgingival microbiome in periodontitis patients before and after treatment at the same tooth sites can serve as a diagnostic and prognostic indicator. 38 genera that had an abundance of more than 1% were identified, Prevotella and Fusobacterium being the most abundant genera. Their results suggested that Synergistetes, Filifactor and Mycoplasma should be considered expanded members of the red complex.

Santigli E et al (2017)[113,143] conducted a study in five 10-year-old children to study the sampling modification effects in the subgingival microbiome profile of healthy children. The 5 major phyla found in all samples were Actinobacteria, Bacteroidetes, Proteobacteria, Firmicutes and Fusobacteria.

Payungporn et al (2017)[99] conducted a study to identify potential bacterial species associated with periodontal disease in ten Thai patients within the age group of 43 to 53 years, of which 5 were from healthy controls and 5 were patients with chronic periodontitis. It was observed that *Porphyromonas gingivalis and Prevotella intermedia* were significantly associated with periodontal disease, whereas other bacteria like *Treponema denticola, Treponema medium, Tannerella forsythia, Porphyromonas endodontalis* and *Filifactor alocis* may be potentially associated with periodontal disease in Thai patients.

VARIOUS ECOLOGICAL NICHES IN THE ORAL CAVITY:

F. nucleatum and *P. intermedia* are involved in the etiology of tonsillitis**.** Even in the edentulous mouth of infants or of denture wearers the proportions of periodontopathogens can be very high excluding *A. actinomycetemcomitans* and *P. gingivalis.*[17] Most periodontopathogens are even able to colonise in the maxillary sinus. **Wald 1998, Caufield & Gibbons 1979**[10,13] assumed that most of the *S. mutans* cells in the saliva or on the tongue are derived from the biofilm present on the teeth and that the mucosae could not act as a reservoir for the colonization of teeth by those organisms.

TRANSLOCATION OF MICRO ORGANISMS:

Christersson et al. (1985)[29,136] demonstrated the translocation of *A. actinomycetemcomitans* via periodontal probes in localised juvenile periodontitis patients. They were able to successfully inoculate previously non-colonized pockets with *A. actinomycetemcomitans* by a single course of probing with a probe previously inserted in a colonized pocket of the same patient. 28 out of the 30 healthy sites (previously negative for this bacterium), sampled immediately after ‘‘inoculation’’, yielded on average 16*À*103 *A. actinomycetemcomitans* cells (versus 105 on the probe). One week later 8 of the 9 sites, which were probed forcefully, remained positive for *A. actinomycetemcomitans*, but at the end of the second week this number was reduced to 1 out of 9.

Kohavi et al. (1994)[54] and Mombelli et al. (1995)[87,59] made comparable observations but however, in the supragingival plaque, the frequency of occurrence and the levels of bacteria considered as late colonisers (*F. nucleatum* and *A. actinomycetemcomitans*) are higher on tooth surfaces compared to implants, suggesting that while the same bacteria populate the implant and tooth surfaces, the number of bacteria and the plaque composition may differ.

Papaioannou et al. (1996)[95] compared the subgingival microbiota around teeth and implants (within the same jaw) from healthy patients to those of chronic adult periodontitis patients or patients with rapidly advancing periodontitis. Both the percentages of spirochetes and motile organisms, and the detection frequency of pathogenic species (*P. gingivalis*, *P. intermedia*, *B. forsythus*; based on DNA-probing) were nearly identical for both abutment types, especially when deeper pockets (>4 mm, an environment closer to diseased sites in the natural dentition) were considered.

Clinical significance of the intra-oral translocation of bacteria, Impact of a full-mouth tooth extraction on distribution of periodontopathogens:

Danser et al. (1995)[17] studied the prevalence of selected periodontopathogens on the oral mucous membranes and in the saliva, it seemed plausible that at least one of the periodontal pathogens such as *A. actinomycetemcomitans*, *P. gingivalis* or *P. intermedia* must have been present before tooth extraction, their detection frequencies were extremely low 0/26

for *A. actinomycetemcomitans*, 2/26 for *P. gingivalis* or 7/26 for *P. intermedia*, respectively.

SALIVA:

Saliva, an oral fluid that contains an abundance of proteins and genetic molecules and is readily accessible *via* a totally non-invasive approach, has long been recognized as the potential solution to these limitations.[64]

Saliva provides an easily available, non-invasive diagnostic medium for a rapidly widening range of diseases and clinical situations.[79,80] Proposed salivary diagnostic markers for periodontal diseases have included serum and salivary molecules such as immunoglobulins, enzymes constituents of gingival crevicular fluid, bacterial components or products, volatile compounds, and phenotypic markers, such as epithelial keratins.[80]

MARKERS AFFECTING THE DENTAL BIOFILM:

SPECIFIC BIOMARKERS:

Immunoglobulins (Ig) are important specific defense factors of saliva. Of the different classes of immunoglobulins, IgA, IgG and IgM influence the oral microbiota by interfering with the adherence of bacteria or by inhibiting bacterial metabolism, with IgA being the predominant immunoglobulin. Patients with periodontal disease are shown to have higher salivary concentrations of IgA, IgG and IgM specific to periodontal pathogens compared with healthy patients.[115] Additionally, the levels of these immunoglobulins in saliva are greatly reduced after periodontal treatment.[104]

As a consequence, the screening of saliva, especially for IgA, has been previously discussed as a useful, noninvasive technique to identify individuals who have the potential to develop periodontal disease or those who are currently responding to a periodontopathogenic infection.[79,80] Biomarkers of saliva are illustrated in **(FIGURE 4)**

Non-specific biomarkers:

Mucins are glycoproteins produced by submandibular and sublingual salivary glands and numerous minor salivary glands. The physiological functions of the mucins (MG1 and MG2) are cytoprotection, lubrication, protection against dehydration and maintenance of viscoelasticity in secretions. The mucin, MG2, affects the aggregation and adherence of bacteria and is known to interact with Aggregatibacter actinomycetemcomitans, and a decreased concentration of MG2 in saliva may increase colonization with this periodontopathogen.[33] Lysozyme is an antimicrobial enzyme with the ability to cleave chemical bonds in the bacterial cell wall. It can lyse some bacterial species by hydrolyzing glycosidic linkages in the cell wall peptidoglycan. It may also cause lysis of bacterial cells by interacting with monovalent anions and with proteases found in saliva. This combination leads to destabilization of the cell membrane, probably as a result of the activation and deregulation of endogenous bacterial autolysins. Patients with low levels of lysozyme in saliva are more susceptible to plaque accumulation, which is considered a risk factor for periodontal disease.[44] Lactoferrin is an iron-binding glycoprotein produced by salivary glands, which inhibits microbial growth by sequestering iron from

the environment, thus depriving bacteria of this essential element. Lactoferrin is strongly up-regulated in mucosal secretions during gingival inflammation and is detected at a high concentration in saliva of patients with periodontal disease compared with healthy patients.[34] Histatin is a salivary protein, in addition to its antimicrobial activities, it is also involved in the inhibition of the release of histamine from mast cells, affecting their role in oral inflammation.[36] Peroxidase is a salivary enzyme produced by acinar cells in the salivary glands. This enzyme removes toxic hydrogen peroxide produced by oral microorganisms and reduces acid production in the dental biofilm, thereby decreasing plaque accumulation and the establishment of gingivitis and caries. Patients with periodontal disease have demonstrated high levels of this enzyme in saliva.[37]

MICROBIOME IN PERIODONTITIS:

De Araujo WC, Macdonald JB[21] found that the predominant subgingival organisms in prepubertal children are reported to be Lactobacillus, Streptococcus, Capnocytophaga, Eikenella, Prevotella and Actinomyces spp., and Peptostreptococcus.

Hajishengallis et al., 2011[38,39], Recent studies in mice demonstrated that, low-levels of colonization by periodontitis-associated microorganisms such as Porphyromonas gingivalis are sufficient to dysregulate innate immunity pathways, facilitate an increase in overall community biomass and trigger periodontal destruction. Recent studies in mice demonstrated that, low-levels of colonization by periodontitis-associated microorganisms such as

Porphyromonas gingivalis are sufficient to dysregulate innate immunity pathways, facilitate an increase in overall community biomass and trigger periodontal destruction **(FIGURE 5)**

MICROFLORA IN SALIVA

R. S. Percival[84], Marsh[11,72,83] found that the prevalence of yeasts in saliva also increased with age, with greater isolation frequencies and viable counts in those aged 80years, and higher proportions in those aged 60 years.

Taubman et al, (2007)[127] have demonstrated that host susceptibility is of primary importance with an as yet uncharacterized defect of the immune system, which causes defects in the regulation of osteoclast recruitment, differentiation and activation, causing affected individuals to mount an inappropriately aggressively inflammatory response to the normal microbiota.

De jong et al (1984)[19], have suggested that microorganisms in dental plaque can survive in saliva, and can utilize salivary components as a substrate. It was shown that saliva could serve as a growth medium for oral *Streptococcus* species and *A. viscous.*

In a study by **De Jong et al (1986)[19],** micro-organisms from supragingival plaque were grown on saliva agar. When supragingival plaque was plated on saliva and blood agar plates, the composition of the microflora isolated from the plates were similar. The authors concluded that the supragingival microflora could utilize saliva as a complete nutrient source.

Asikainen et al. (1991)[5] compared the recovery of *A. actinomycetemcomitans* from subgingival sites, the dorsum of the tongue and

saliva. When *A. actinomycetemcomitans* was recovered from subgingival sites it was also found in 69.9% and 35.9% of the samples of stimulated and unstimulated saliva, respectively.

Umeda et al. (1998)[111] examined the presence of periodontopathic bacteria in whole saliva in relation to occurrence of the microorganisms in subgingival plaque. Using polymerase chain reaction, the presence of *P. gingivalis, Prevotella intermedia* and *T. denticola* in whole saliva and in periodontal pocket samples along with *Prevotella nigrescens.*

A highly significant correlation was found between *S. mutans* level in dental plaque and the salivary level of this microorganism.

Using periodontal pocket samples as the reference, bacterial detection in whole saliva had a sensitivity of 42.6% for *A. actinomycetemcomitans*, 68.4% for *Bacteroides forsythus*, 97.8% for *P. gingivalis*, and 88.7% for *P. intermedia*. The specific of bacterial detection for these micro-organisms in saliva was 88.5%, 71.2%, 77.9% and 77.1% respectively.[76] The large increase in the number of bacteria on the teeth was reflected by an increase in salivary counts of *Actinomyces* species.

Salivary levels of *A. actinomycetemcomitans, P. gingivalis, P.intermedia, Campylobacter rectus,* and *Peptostreptococcus micros* were determined by bacterial culture and related to clinical periodontal status in 40 subjects with varying degrees of periodontitis.[135]

DETECTION AND ENNUMERATION OF BACTERIAL SPECIES FROM PERIODONTAL SAMPLES:

Microscopic studies

In 1683 **Antonio van Leeuwenhoek** conducted a study in which he studied the scrapings from human teeth using microscope and described 5 different types of animalcules. **Coyler** described the evaluation of pocket microorganisms using dark microscope. **Theodore Rosebury**[107] isolated bacteria of etiological importance in periodontal disease. **Keyes (1965)**[50] used phase contrast microscopy in periodontal diagnosis to identify bacterial morphotypes.

Culture based methods

Cultivation of organisms and identification of species by their phenotypic traits was one of the major techniques used by researchers to identify plaque bacteria. **Moore & Moore**[88] examined composition of subgingival plaque samples from periodontal healthy subjects and different periodontal disease states employed cultural techniques to examine over 17,000 isolates from over 600 periodontal sites. Although this was a massive amount of work, it was considered a limited number of samples by current standards. The major strength of this culture was that majority of the bacterial species examined can be grown and identified in lab conditions. The main drawback of this culture method was its narrow spectrum, and it was regarded as a time-consuming and expensive undertaking because only few plaque samples in small numbers of subjects can be examined.

Studies by **Kolenbrander (2000)[24,55], Vartoukian et al (2007)[26]** and **Siqueria et al (2010)[136]** have estimated that 50% to 60% of distinct bacterial phyla which is present in the oral cavity still have no cultivable representatives. However, to assess the bacterial sensitivity to antibiotics, cell culture was considered as a mandatory procedure.

Immunologic and enzymatic assays

To enumerate the specific species of microorganisms in the oral cavity without their cultivation, Immunofluorescence techniques and enzyme-linked immunosorbent assay (ELISA) techniques are most commonly used which are antibody-based methods; it is dependent on specificity of developed antibodies to specific taxa. They are rapid and less expensive than culture. However, they are limited to species for which reagents have been developed. Disadvantage is that it is difficult to use these techniques to evaluate species in large numbers of plaque samples and moreover, it is time-consuming to develop and validate specific antisera to new species.

DNA – DNA hybridization or checkerboard

DNA-DNA hybridization is a molecular approach which is used to detect bacteria based on hybridization of target species to labelled genomic DNA that has been attached to nylon membranes. Studies by **Loesche WJ et al[74], Haffajee[122],** and **Socransky[120]** have reported on levels of limited number of species with this method in adult periodontitis, periodontal health, refractory periodontitis and response to therapy. This method provides a major

benefit for studies of oral microbial ecology due to advantages like detection of multiple species from each sample simultaneously, and study of large sample size for large numbers of species. Checkerboard technique is rapid, sensitive, and relatively inexpensive but is also dependent on culture technique to cultivate the target species for creating genomic probes. Like antibody-based assays, cross reactivity can be verified only with cultivated species hence specificity of the probe is an unknown variable.

Polymerase chain reaction

Kary Mullis[89] first developed polymerase chain reaction (PCR) technique to amplify specific genes or parts of genes which are then used to identify bacterial species from which they originated. In a study by **Kumar PS et al (2005)**[32,61] species-specific PCR primers were designed and used in individual PCR reactions to detect prevalence of target species in plaque samples of healthy and diseased subjects. These studies have concluded that several species, including uncultivated ones, were associated with oral health or periodontitis. Given the appropriate primers, this method is rapid and simple, can detect small numbers of cells of a given species, and indicates the presence or absence of a species in the sample. It has certain disadvantages of not providing quantitative data, may not be cost effective for large sample sizes, and for applications where relative levels of species are important, PCR may not be ideal.

DNA Probes

Oligonucleotide probes are short probes. They are highly specific and likelihood of cross-reactions with other species is very low. They are targeted to a limited segment of DNA of an organism. Oligonucleotide probes tend to be less sensitive for detection of low numbers of bacteria than whole genomic probes.

Whole genomic DNA probes are constructed using the entire genome of a bacterial species as the target and thus can be quite sensitive. The use of the entire genome may increase the probability of cross-reactions between species because of common regions of DNA among closely related species. The technique can detect only species for which DNA probes have been prepared..

OPEN ENDED APPROACHES- 16S rRNA sequencing analysis:

Open ended approaches allow identification of even uncultivated and previously unknown species. According to **Spratt (2004)**[124] these approaches are based on 16 S rRNA sequencing, amplification and analysis of the 16S rRNA genes in a microbiome sample. 16S rRNA has proven to be the most useful phylogenetic marker to identify bacteria and to determine their evolutionary relationships. The hypervariable regions are used as signatures which are used to discriminate one species from another. 16S rRNA gene is large enough (about 1500 bases) to provide sufficient sequence variability among bacteria, which enables them for the comparisons possible at different taxonomic levels.

DNA and protein sequencing started in the 1970s when the virus Lambda (50,000 nucleotides) was sequenced by **Sanger et al, Frederick Sanger**[3] and colleagues described the use of chain-terminating di-deoxynucleotide analogues that caused base-specific termination of primed DNA synthesis and this came to be popularly known as Sanger sequencing method which was considered the gold standard, and over the years, whole genome sequencing of many bacteria has been carried out using this method.

Over the past decade, next generation sequencing technologies have emerged, which are of high standards and are able to generate three to four orders of magnitude more sequences and are also relatively less expensive.

NEXT GENERATION SEQUENCING TECHNOLOGY (NGS):

Next generation sequencing methods deploy a wide spectrum of technologies such as colony synthesis, sequencing by synthesis, sequencing by ligation and single molecule DNA sequencing. NGS is performed by machinery automated cyclical ligation of oligonucleotides or by repeated cycles of polymerase-mediated nucleotide extensions.

Fundamentals of NGS platforms:

NGS platforms share a common technological feature—massive parallel sequencing of clonally amplified or single DNA molecules that are spatially separated in a flow cell. This design is a paradigm shift from that of Sanger sequencing, which is based on electrophoretic separation of chain-termination products produced in individual sequencing reactions. In NGS, sequencing is performed by repeated cycles of polymerase-mediated nucleotide extensions

or, in one format, by alternative cycles of oligonucleotide ligation. As a massively parallel process, NGS generates hundreds of megabases to gigabases of nucleotide sequence output in a single instrument run, depending on the platform. The 2 basic procedures on which this NGS runs are ligation of DNA fragments with oligonucleotide adaptors and fragment immobilization to a solid surface, such as a bead.

The three commonly used platforms for massive parallel DNA sequencing at present are the Roche/454 FLX (Life Sciences, Branford, CT, **Margulies et al**)[81], the Illumina/ Solexa Genome Analyzer (Illumina, **San Diego, CA, Bentley DR, 2006**, **Korbel et al.,**)[56] and the Applied Biosystems/ SOLidD (Life Technologies, Carlsbad, CA). The most recent powerful NGS platforms have significant reductions in the run time and remarkable data output, they include HiSeq and the Ion Torrent Personal Genome Machine (PGM).

ROCHE 454 LIFE SCIENCES SYSTEM

The 454 technology is basically derived from technological convergence of pyrosequencing and emulsion PCR. In **2000**, **Jonathan Rothberg**[52] founded 454 Life Sciences, which developed the first commercially available NGS platform, GS 20, which was launched in 2005. One of the major drawbacks of this system is that sometimes more than one nucleotide is incorporated in DNA template during a cycle, making it difficult to resolve homopolymeric stretches of sequence (e.g. CCCCC or AAAAA). **(FIGURE-6)**

ILLUMINA/SOLEXA GENOME ANALYZER

In 1997, British chemists Shankar Balasubramanian and David Klenerman conceptualized an approach for sequencing single DNA molecules attached to microspheres and founded Solexa in 1998. The Solexa Genome Analyzer, the first ―short read‖ sequencing platform, was commercially launched and acquired by Illumina in 2006. Genome Analyzer uses a flow cell with bound oligonucleotide anchors wherein template DNA is fragmented into several hundred base pairs and end-repaired**. (FIGURE-7)**

APPLIED BIOSYSTEMS SOLiD:

The SOLiD (Supported Oligonucleotide Ligation and Detection) System 2.0 platform is a short-read sequencing technology which is based on ligation. This approach was developed in the laboratory of George Church and was reported in 2005 along with resequencing of *Escherichia coli* genome. This system can generate 4 GB of sequence but the reads are only 35 nucleotides. The weakness of SOLiD system is that it yields biased sequence coverage in AT-rich repetitive sequences and only 35% of the raw reads are useable, compared with 95% for the 454 system. Another disadvantage is that it requires long run times.

THE HUMAN ORAL MICROBIOME DATABASE

Research over the past 20 years has focused on defining breadth and diversity of oral microbiome by obtaining 16S rRNA gene sequence information for both cultivable and as yet uncultivated oral bacteria. The majority of bacterial species isolated from the oral cavity are included in 4 of

the 10 bacterial phyla; Phylum 1 (Proteobacteria), Phylum 2 (the gram-positives), Phylum 5 (the spirochetes) and Phylum 6 (the flavobacter-bacteroides group). There are no known human oral representatives from the other 6 phyla. Though human oral microbiome is the most studied human microflora, 53% of species have not been named yet and 35% of species are uncultivated. The uncultivated taxa are identified mainly by 16S rRNA sequence information.

The ***Human Oral Microbiome Database (HOMD)*** is a specifically designed database to provide a provisional naming scheme where each oral taxon is given a Human Oral Taxon (HOT) number linked to comprehensive information and tools for examining and analyzing each taxon in the human oral microbiome at both taxonomic and genomic level. This dynamic database provides a curated taxonomy of oral prokaryotes, a curated set of full-length 16S rRNA reference sequences and BLAST tools that allow identification of unknown isolates or clones based on their 16S rRNA sequence; additionally, phenotypic, bibliographic, clinical and genomic information are linked for each taxa. Organisms of the human oral cavity are organized in a taxonomy hierarchy, which leads to individual pages for every oral taxon with comprehensive information and links. The genomic component of HOMD contains both static and dynamically updated annotations as well as bioinformatics analysis tools for all the genomic sequences, and curated 16S rRNA gene reference sequences for all human oral microbes. HOMD may serve as an example of a body site-specific tool for other communities.

More recently, a similar database was set up by **Griffen A et al**[32] known as CORE, a phylogenetically curated 16S rDNA database of the core oral microbiome, which offers improved and more robust identification of human oral bacterial 16S rRNA gene sequences compared with other methods. Its main goal is to provide a comprehensive and minimally redundant collection of oral bacteria at the genus and species level, as well as providing support for inferring community divergence and analysis of large datasets.

The basic list of oral bacteria came from the literature works of **Dzink JL**[27]**, Sockransky**[121]**, Tanner**[125] and **Moore WE**[88]. In 2010, Dewhirst identified 1,179 taxa of which 24% were named, 8% were cultivated but unnamed, and 68% were uncultivated phylotypes. Upon validation, 434 novel non-singleton taxa were added to the HOMD. **Liu B et al**[70] reported using a metagenomic approach by applying next-generation sequencing to sequence entire microbial DNA within a sample directly, and reconstructing genomes of microbiota via de novo assembly or mapping against a reference genome database. According to **Blainey P**[9]**,** the emerging field of single-cell genomics is also currently being implemented for bacteria and Archaea. The issue with these commercially available tests is the question of their true value in terms of reliability for detecting causative agents of disease, given our limited knowledge of the complex ecosystem involved. The other major concern lies in the ability of clinician or diagnostic company to interpret results correctly and in such a way as to provide benefit for patients.

STUDIES ON THE ANALYSIS OF PLAQUE MICROBIOME

The proven microbiological etiology of periodontal diseases is the rationale for application of various methods for microbiological identification, in order to accomplish better diagnosis and for targeted anti-infectious periodontal treatment. Various microorganisms inhabiting the periodontal environment and their complex relationships were presented by Socransky and Haffajee and were united in periodontal complexes. The first complete sequence of a microbial genome was published in **1995** by **Fleischmann RD et al**[28]. In the past 50 years, numerous studies by **Paster et al**[15,97,98]**, Baker et al, Kumar et al**[32,61] **and Aas et al**[98] have characterized the community composition of oral microbiota. Using culture-dependent and independent methods, estimates of oral biodiversity have implicated more than 700 different microbial species. The composition of subgingival microbiota of chronic periodontitis in adults has been described by culture immunological and molecular methods. Culture analysis of subgingival plaque samples of early periodontitis by **Tanner et al**[18,125] detected *Tannerella forsythia, Campylobacter rectus, and Selenomonas noxia* associated with progressing disease pattern compared with non-progressing disease subjects, whereas *Porphyromonas gingivalis* was associated, by whole genomic DNA probes, with progressing periodontitis. These species have also been associated with moderate and advanced periodontitis. Molecular PCR cloning and sequencing methods have identified several species that are rarely or not detected by culture methods, some of which show strong associations with adult

periodontitis. **Aas J et al[98]** in a study in **2005** established that there is a distinctive predominant bacterial flora of the healthy.

oral cavity that is highly diverse, as well as site and subject specific. They found 141 predominant species of which 60% have not been cultivated. 13 new phylotypes were identified, and species typically associated with periodontitis were not detected.

Tanner AC et al[125] conducted a cross-sectional evaluation of 141 healthy and periodontitis individuals to compare microbiota of subgingival and tongue samples between early periodontitis and health using oligonucleotide probes and PCR. *Porphyromonas gingivalis and Tannerella forsythia* were associated with early periodontitis by direct PCR, and they found that microbiota of tongue samples was less sensitive than that of subgingival samples in detecting periodontal species.

Several studies have employed next-generation sequencing technologies to analyze the species richness of the oral microbiota. Estimates from one of these studies by **Keijser et al (2008)**[49] suggested that up to 19,000 phylotypes may exist in human oral cavity. **Keijser[49]** conducted a study for pyrosequencing analysis of oral microflora from saliva and supragingival plaque in 71 and 98 healthy individuals respectively using 454 Life sciences and Genome Sequencer 20 system. His results generated 19,000 phylotypes representing 22 taxonomic phyla and 3621 and 6888 species-level phylotypes in saliva and plaque respectively. He showed that the vast majority (namely 99.6%) of sequences in saliva and subgingival plaque samples of adults belong

to one of the seven major phyla: *Actinobacteria, Bacteroides, Firmicutes, Fusobacteria, Proteobacteria, Spirochetes, or candidate division TM7.*

G Xie et al[139] reported a metagenomic analysis of a healthy human plaque sample using a combination of second generation sequencing platforms, and revealed the presence of 12 well-characterized phyla, members of the TM-7 and BRC 1 clade, and unclassified sequences. 73% of the total assembled counting sequences were predicted to code for proteins, 2.8% of the predicted genes coded for proteins involved in resistance to antibiotics and toxic compounds.

Liu B et al[70] performed a pilot study in 2012 to analyze the global genetic, metabolic and ecological changes associated with periodontitis in 15 subgingival plaque samples from two periodontitis patients and three healthy individuals using metagenomics. They found that the disease samples shared a similar bacterial species cluster that was different from the completely healthy samples suggesting that the disease state occupied a narrow region within the space of possible configurations of the oral microbiome. They observed a shift in the oral bacterial composition from a gram-positive dominated community in the healthy subject to a gram-negative dominated community in periodontal disease. The shift in bacterial species from gram-positive to gram-negative confirmed previous findings using different molecular biological methods. Liu and colleagues also observed higher bacterial diversity in the diseased samples than in the healthy samples, which confirmed results obtained using 16S rRNA sequence analysis.

Griffen et al[32] conducted a study using 454 pyrosequencing of 16S rRNA genes and identified and reported 16 phyla, 106 genera and 596 species. Community diversity was higher in disease, 123 species were significantly abundant in disease and 53 species in health. *Spirochaetes, Synergistetes and Bacteroidetes* were more abundant in disease whereas *Proteobacteria* were found in higher levels in healthy controls. Within the phylum *Firmicutes*, the class Bacilli was health associated whereas *Clostridia, Negativicutes and Erysipelotrichia* were associated with disease.

Abusleme et al[1] conducted a study in 2013 to and found a higher biodiversity and biomass in periodontitis compared to health, with periodontitis having higher proportions of *Spirochetes, Synergistetes, Firmicutes and Chloroflexi;* while the proportions of *Actinobacteria* like Actinomyces were increased in health. They also showed an association between biomass and community structure in periodontitis with proportions of specific taxa correlating with bacterial load.

Trajanoski et al[113] conducted a study to demonstrate pyrosequencing data processing for the characterization of the subgingival microbiome in healthy children and reported 2617 operational taxonomic units (OTU) that were classified into 11 phyla with the majority accounted for by *Bacteroidetes* (27.24%), *Actinobacteria* (14.21%), *Firmicutes* (17.92%), *Proteobacteria* (10.85%), *Spirochaetes* (4.09%) and *Fusobacteria* (3.59%) in the subgingival samples.

Wang J and colleagues[42] they analyzed periodontal samples and found a strong correlation between bacterial community structure and disease status, and identified numerous novel microbial inhabitants. The 4 most abundant phyla were *Bacteroidetes* (41-59.2%), *Actinobacteria* (9.3-41%), *Proteobacteria* (5.2-40.1%) and *Firmicutes* (14.8-58.3%). They also examined Fim A type, an important biofilm gene involved in interactions of *Porphyromonas gingivalis* with other microorganisms. They found that the most prevalent P. gingivalis FimA was type II, which is consistent with previous studies.

Xiuchun Ge et al[140] examined the subgingival bacterial biodiversity in 88 untreated chronic periodontitis patients by comparing the oral microbiome in deep (diseased) and shallow (healthy) sites. 51 of 170 genera and 200 of 746 species were found significantly different in abundances between the 2 sites. They also reported that this difference was influenced by patient level effects such as clinic location, race and smoking.

In a study by **Yan Li et al**[64] they examined the phylogenetic and functional gene differences between 25 periodontal and 12 healthy subjects using MiSeq sequencing. 39 genera were significantly different between healthy and periodontitis group and *Fusobacterium, Porphyromonas, Treponema, Filifactor, Eubacterium, Tannerella, Hallella, Parvimonas, Peptostreptococcus and Catonella* showed higher relative abundances in the periodontitis groups. A variety of genes involved in virulence factors, amino acid metabolism and glycosaminoglycan and pyrimidine degradation were

enriched in periodontitis whereas genes involved in amino acid synthesis and pyrimidine synthesis exhibited a significantly lower relative abundance compared with healthy group.

Shi B et al[117] aimed to determine whether dynamic changes in the subgingival microbiome in periodontitis patients before and after treatment at the same tooth sites can serve as a diagnostic and prognostic indicator. 38 genera that had an abundance of more than 1% were identified, *Prevotella* and *Fusobacterium* being the most abundant genera. Their results suggested that *Synergistetes, Filifactor and Mycoplasma* should be considered expanded members of the red complex.

Hong BY et al[2] conducted a study to explore the existence of different community types in periodontitis and their relationship with host demographic, medical and disease-related clinical characteristics. Their results suggested 2 types of communities (A and B) existed in periodontitis. Type B communities harbored greater proportions of certain periodontitis associated taxa like *P. gingivalis, T. forsythia and T. denticola* and other recently linked periodontitis associated ones. In contrast, type A communities had increased proportions of different species and were also enriched for health associated species and core taxa.

Zheng et al[146] performed a study to analyze the microbial characteristics of oral plaque around implants using pyrosequencing of 16S rRNA gene, and reported an increase in microbial diversity in subgingival sites of ailing implants compared with healthy implants. Periodontal pathogens

like *P. gingivalis, T. forsythia and P. intermedia* were clustered into modules in the peri-implant mucositis network.[146]

Kotsilkov (2015)[59] compared the diagnostic potential of microbiologic culture and real-time PCR identification for detection of putative periodontopathic bacteria of 60 patients from deep periodontal pockets with probing depth of more than 7mm. Their results yielded a statistically significant higher detection levels and better diagnostic capability of the RT-PCR, whereas the culture analysis detected only 3 pathogens.[59]

FIGURE-1 PSD MODEL OF PERIODONTAL DISEASE ETIOLOGY

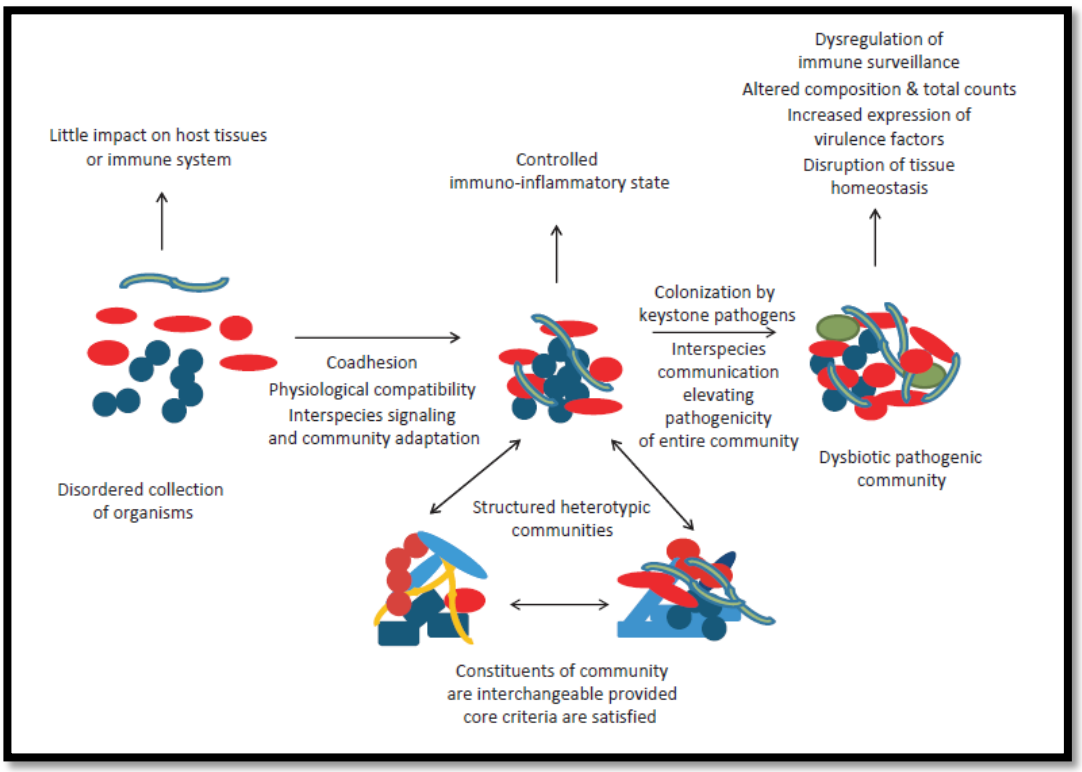


Figure-2: SCHEMATIC ILLUSTRATION OF METABOLIC INTERACTION AMONG DIFFERENT BACTERIAL SPECIES FOUND IN PLAQUE, AND BETWEEN THE HOST AND PLAQUE BACTERIA

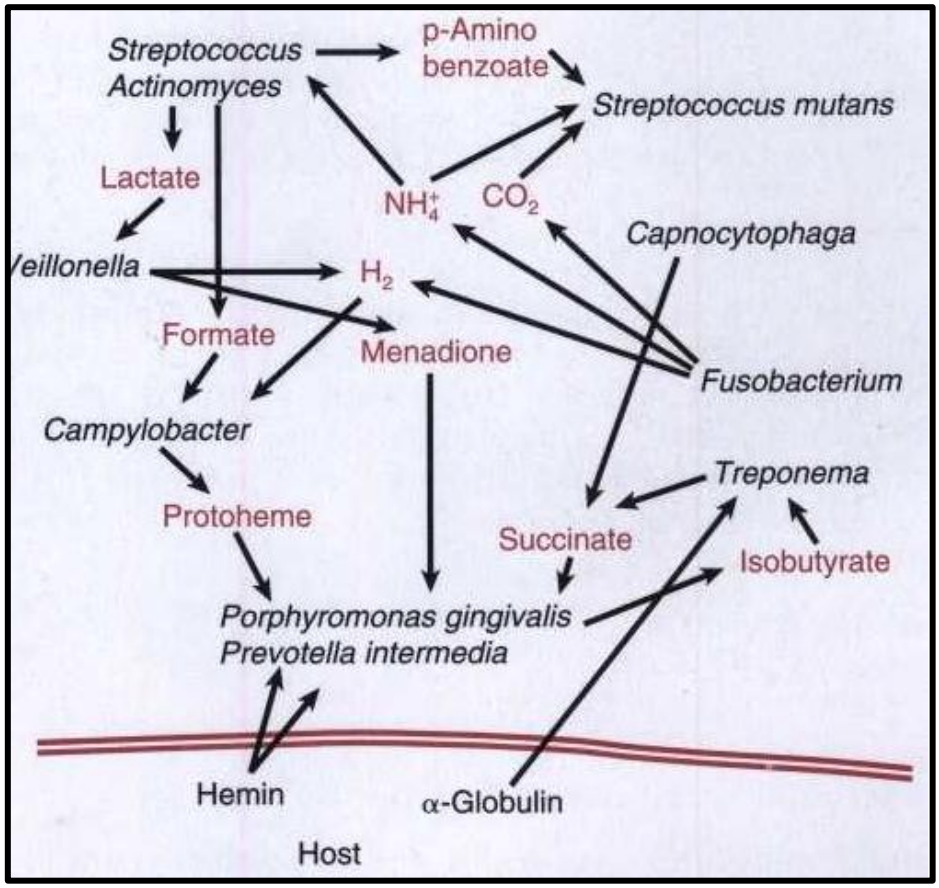


FIGURE 3: BACTERIAL COMPLEXES

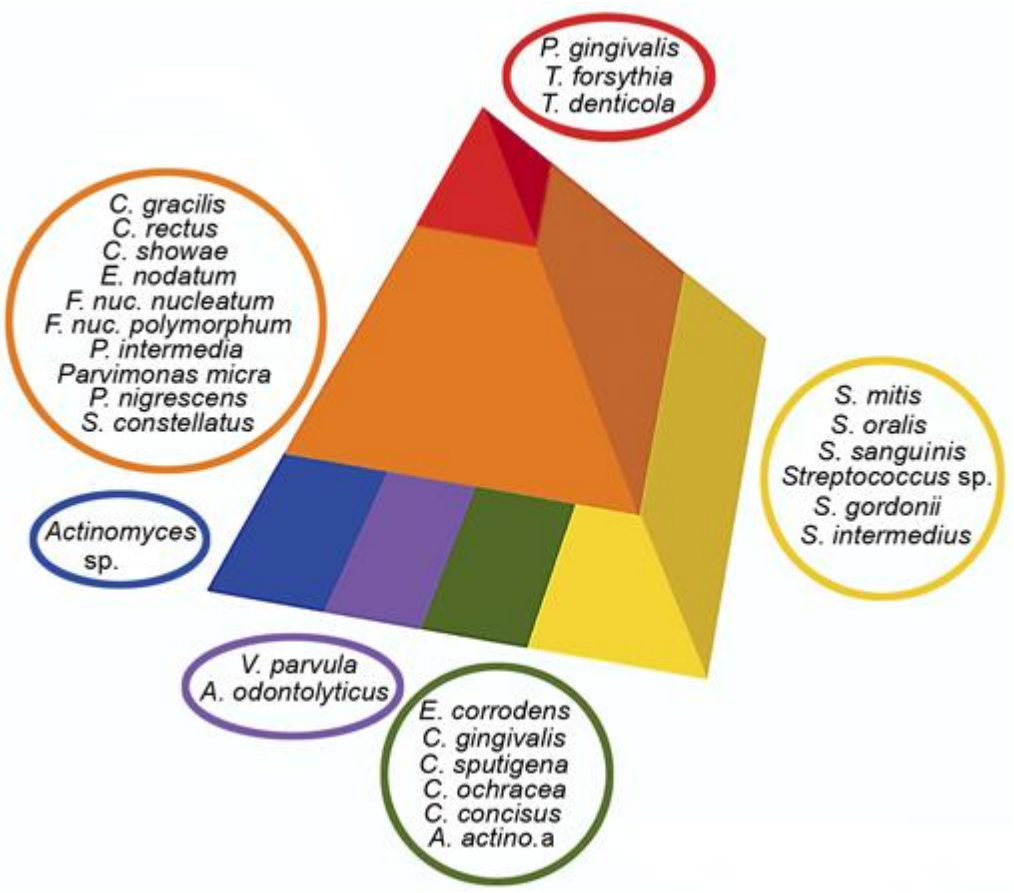


FIGURE 4: MARKERS OF PERIODONTAL DISEASE

Marker	Relationship with periodontal disease	Type of periodontal disease
Specific		
Immunoglobulins (IgA, IgM, IgG)	Interfere in adherence and bacterial metabolism / increased concentration in saliva of periodontal patients	Chronic and aggressive
Nonspecific		
Mucins	Interfere with the colonization of *Aggregatibacter actinomycetemcomitans*	Aggressive
Lysozyme	Regulates biofilm accumulation	Chronic
Lactoferrin	Inhibits microbial growth / increased correlation with *A. actinomycetemcomitans*	Aggressive
Histatin	Neutralizes lipopolysaccharide and enzymes known to affect the periodontium	Chronic and aggressive
Peroxidase	Interferes with biofilm accumulation / increased correlation with periodontal patients	Chronic
Systemic		
C-reactive protein	Increased concentration found in serum and saliva of periodontal patients	Chronic and aggressive

FIGURE 5: CORE SUBGINGIVAL MICROBIOME IN HEALTH AND PERIODONTITIS

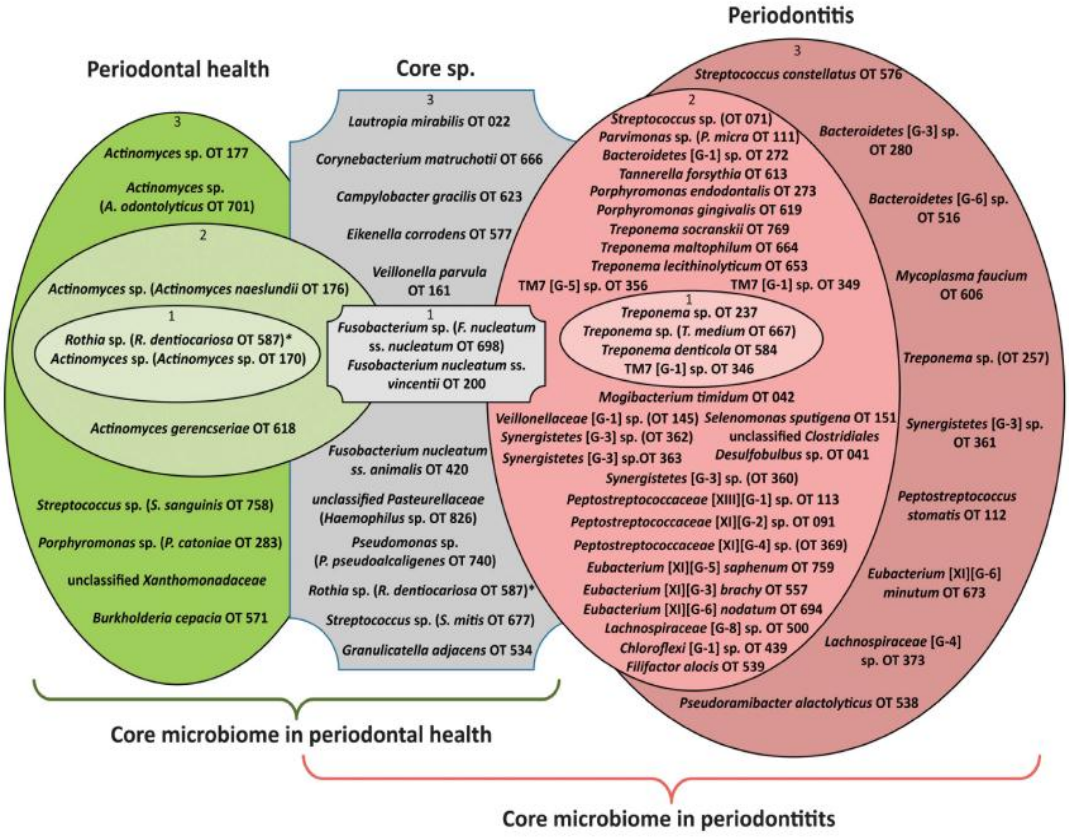


FIGURE 6: ROCHE 454 LIFE SCIENCES SYSTEM

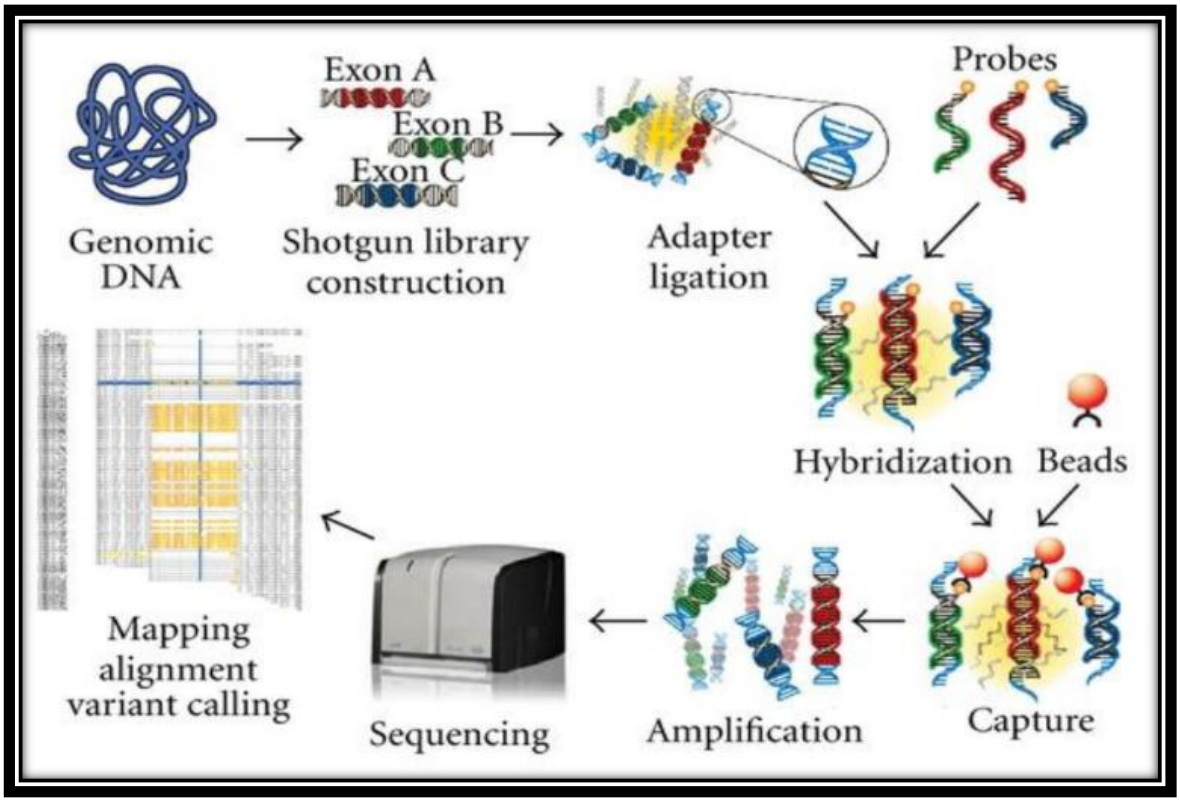
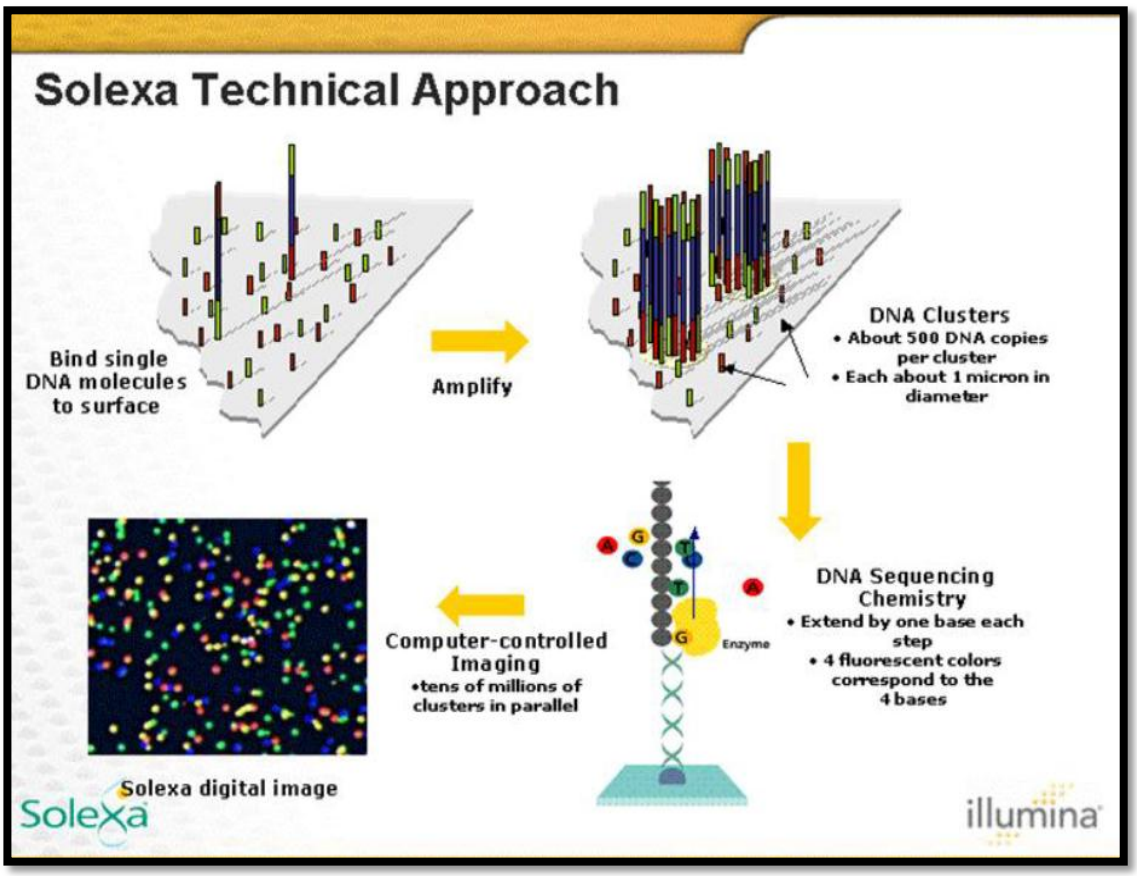


FIGURE 7: ILLUMINA SOLEXA GENOME ANALYZER



Materials & Methods

MATERIALS AND METHODS

Study population

A total of 20 individuals seeking dental treatment in Ragas Dental College and Hospitals, Chennai, were involved in the present study, of which 10 were periodontally healthy individuals (control group) and 10 were chronic periodontitis patients (test group). A diagnosis of chronic periodontitis was determined based on the American Academy of Periodontology parameters.

Control Group consisted of 10 subjects with clinically non-inflamed, healthy gingiva (probing pocket depth {PPD} ≤ 3mm, no clinical attachment loss {CAL}, no bleeding on probing {BOP}).

Test Group consisted of 10 subjects with chronic periodontitis with PPD ≥ 5mm and CAL ≥ 3mm in at least six sites.

The study protocol was explained, and written informed consent was received from each individual before clinical periodontal examinations and saliva sampling. Medical and dental histories were obtained.

INCLUSION CRITERIA

- Subjects exhibiting good general health
- Subjects meeting the criteria of periodontal health and disease as described above were included in this study.

EXCLUSION CRITERIA

- Patients with systemic disorders, such as diabetes mellitus or immunological disorders, HIV
- Patients on drugs that have potential to interfere with microbial characteristics such as immunosuppressant drugs or steroids.
- Patients with history of tobacco usage.
- Patients with history of periodontal treatment in the past 6 months.
- Patients under antimicrobial therapy for the past 6 months.

Saliva sampling

All examinations were performed by a single, calibrated examiner. The periodontitis patients were selected and the sample was collected in a sterile salivary tub. Unstimulated whole saliva was collected in the morning and subjects had to refrain from eating, drinking, smoking or performing any oral hygiene for at least 2 hours prior to the collection. The samples obtained were frozen and stored at -80°C until the sample collection period was completed. All the samples were collected within 2 days and then sent for processing so as to avoid any degradation.

DNA extraction, 16S rRNA amplification, library construction and sequencing

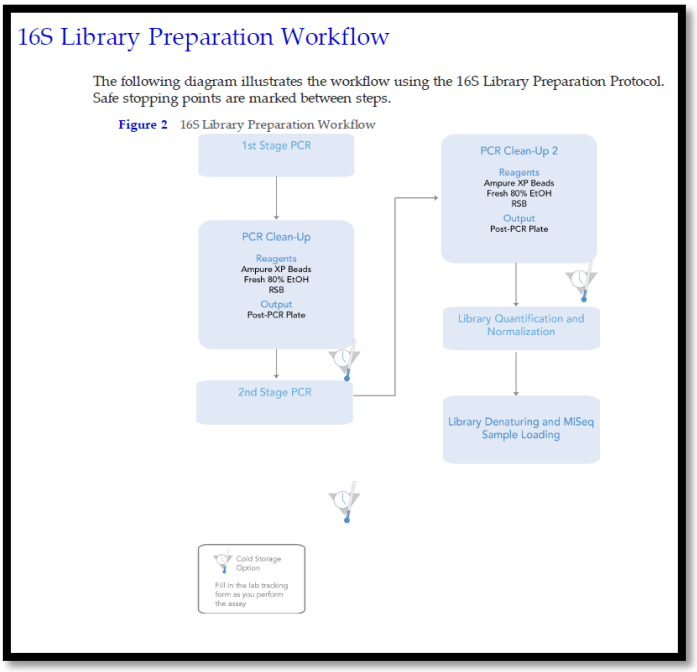
Genomic DNA was extracted from 20 saliva samples of periodontitis and health patients with the Qiagen powersoil kit according to manufacturer's recommendations.

DNA QUALITY CONTROL:

DNA samples were quantitated using Nanodrop. All the samples passed QC and were taken for further library preparation.

16S Metagenomic Sequencing Library Preparation

Preparing 16S Ribosomal RNA Gene Amplicons for the Illumina MiSeq System.



Metagenomic studies are commonly performed by analyzing the prokaryotic 16S ribosomal RNA gene (16S rRNA), which is approximately 1,500 bp long and contains nine variable regions interspersed between conserved regions. Variable regions of 16S rRNA are frequently used in phylogenetic classifications such as genus or species in diverse microbial populations.

This study used the variable V3 and V4 regions of the 16S rRNA gene. After sequencing the V3 and V4 regions a benchtop sequencing system, on-board primary analysis, and secondary analysis using MiSeq Reporter or Base Space, provides a comprehensive workflow for 16S rRNA amplicon sequencing.

Workflow Summary:

1. Order amplicon primers–The protocol includes the primer pair sequences for the V3 and V4 region that create a single amplicon of approximately ~460 bp. The protocol also includes overhang adapter sequences that must be appended to the primer pair sequences for compatibility with Illumina index and sequencing adapters.
2. Prepare library–The protocol describes the steps to amplify the V3 and V4 region and using a limited cycle PCR, add Illumina sequencing adapters and dual-index barcodes are added to the amplicon target. Using the full complement of Nextera XT indices, up to 96 libraries can be pooled together for sequencing.

3. Sequence on MiSeq–Using paired 300-bp reads, and MiSeq v3 reagents, the ends of each read are overlapped to generate high-quality, full-length reads of the V3 and V4 region in a single 65-hour run. The MiSeq run output is approximately > 20 million reads and assuming 96 indexed samples, can generate > 100,000 reads per sample, commonly recognized as sufficient for metagenomic surveys.
4. Analyze on MSR or BaseSpace- The Metagenomics workflow is a secondary analysis option built into the MiSeq Reporter (on-system software) or available on BaseSpace (cloud-based software). The Metagenomics Workflow performs a taxonomic classification using the Greengenes database showing genus or species level classification in a graphical format.

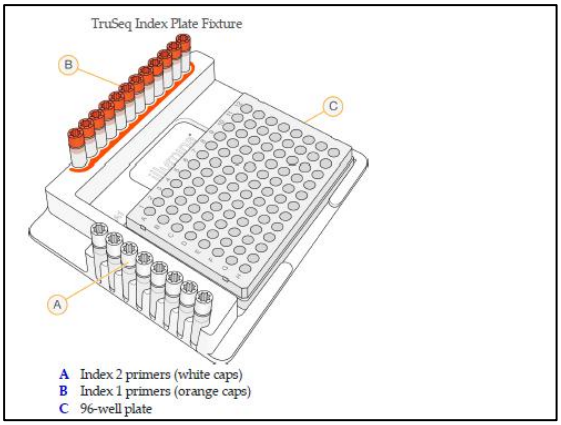
This protocol can be used to sequence alternative regions of the 16S rRNA gene and for other targeted amplicon sequences of interest. When using this protocol for amplicon sequencing other than 16S rRNA, use the Generate FASTQ Workflow (secondary analysis option).

`

AMPLICON PCR:

Reactions were cleaned up with Agencourt AMPure XP beads (Beckman Coulter Genomics) according to the manufacturer's protocol. Attachment of dual indices and Illumina sequencing adapters was performed using 5µl of amplicon PCR product DNA, 5µl of Illumina Nextera XT Index 1 Primer (N7xx) from the

Nextera XT Index kit, 5 µl of Nextera XT Index 2 Primer (S5xx), 25 µl of 2x KAPA HiFi HotStart Ready Mix, and 10µl of PCR-grade water (UltraClean DNA-free PCR water; MO BIO Laboratories, Inc., Carlsbad, CA, USA), with thermocycling at 95°C for 3 minutes, followed by 8 cycles of 95°C for 30 seconds, 55°C for 30 seconds, and 72°C for 30 seconds, and a final extension at 72°C for 5 minutes



Library Quantification, Normalization, and Pooling:

Following Illumina's recommendation quantifying libraries with a fluorometric quantification method that used dsDNA binding dyes was utilised in this study.

The concentrated final library was diluted using Resuspension Buffer (RSB) or 10 mM Tris pH 8.5 to 4 nM. 5 µl of diluted DNA was aliquoted from each library and mixed for pooling libraries with unique indices. Depending on coverage needs, up to 96 libraries can be pooled for one MiSeq run.

For metagenomics samples, >100,000 reads per sample is sufficient to fully survey the bacterial composition. This number of reads allows for sample pooling to the maximum level of 96 libraries, given the MiSeq output of > 20 million reads.

Library Denaturing and MiSeq Sample Loading:

In preparation for cluster generation and sequencing, pooled libraries are denatured with NaOH, diluted with hybridization buffer, and then heat denatured before MiSeq sequencing. Each run must include a minimum of 5% PhiX to serve as an internal control for these low diversity libraries. Illumina recommends using MiSeq v3 reagent kits for improved run metrics.

MiSeq Reporter Metagenomics Workflow:

After samples are loaded, the MiSeq system provides on- instrument secondary analysis using the MiSeq Reporter software (MSR). MSR provides several options for analysing MiSeq sequencing data. For this demonstrated 16S protocol, select the Metagenomics workflow.

By following this 16S Metagenomics protocol, the Metagenomics workflow classifies organisms from your V3 and V4 amplicon using a database of 16S rRNA data. The classification is based on the Greengenes database (http://greengenes.lbl.gov/). The output of this workflow is a classification of reads at several taxonomic levels: kingdom, phylum, class, order, family, genus, and species.

Data analysis was done by using 16s metagenomics tool from Base Space Onsite. Operational taxonomic units (OTUs) were assigned to each sequence using HOMD database.

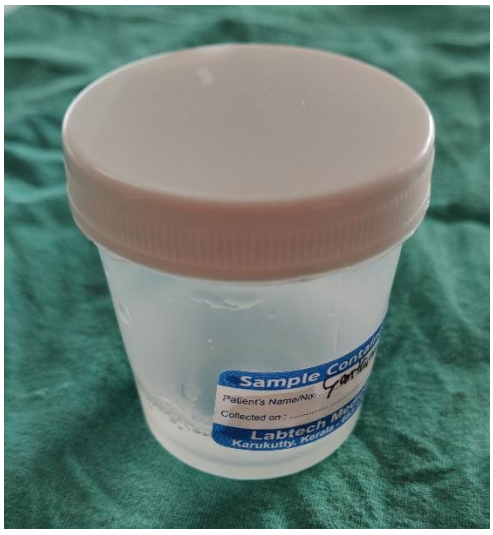
Statistical analysis was performed for individual bacteria using frequency distribution and intergroup comparison was done using Mann Whitney U test with statistical significane set as $P < 0.05$.

Photographs

HEALTH

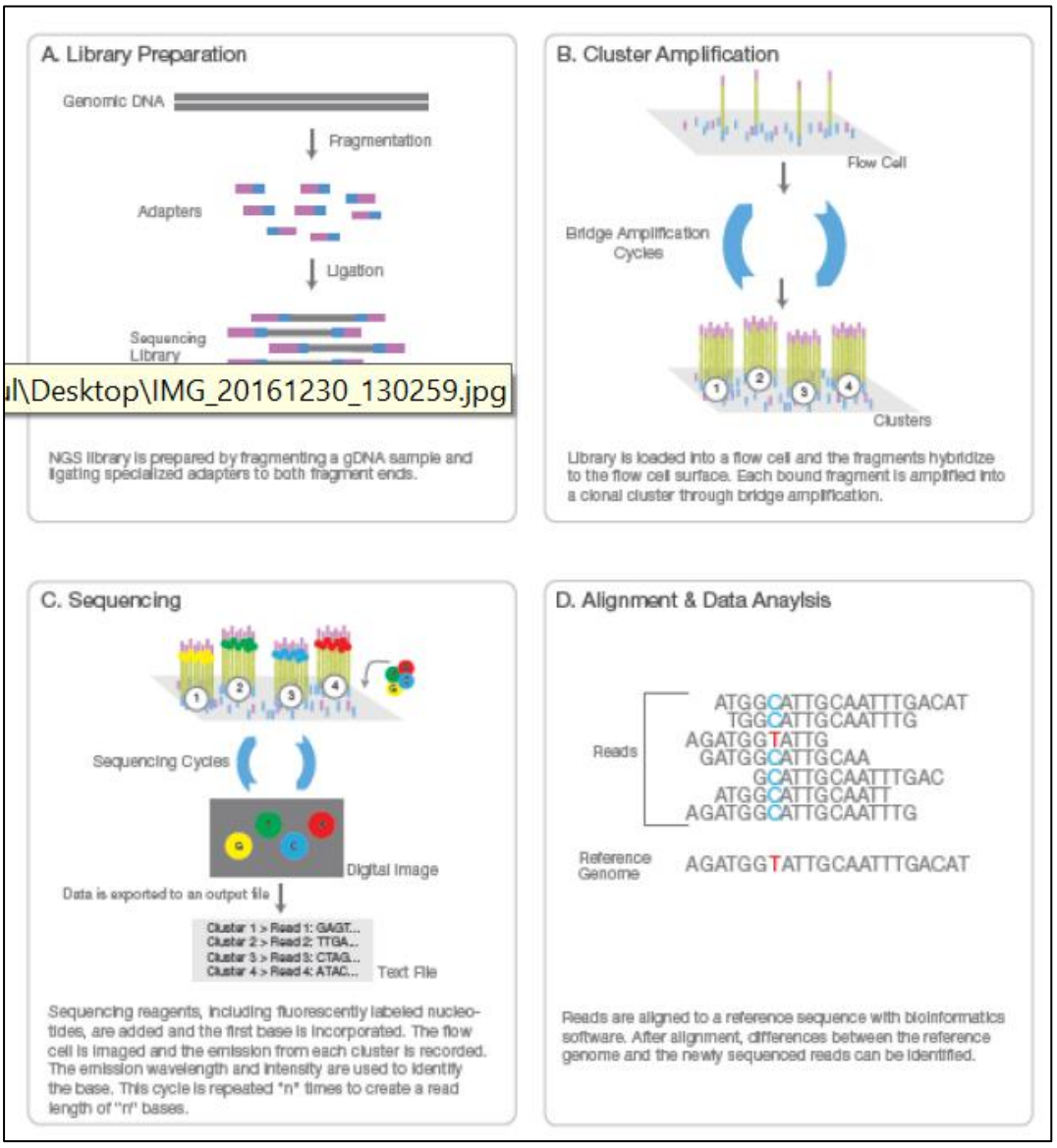
PERIODONTITIS

STERILE SALIVA TUB

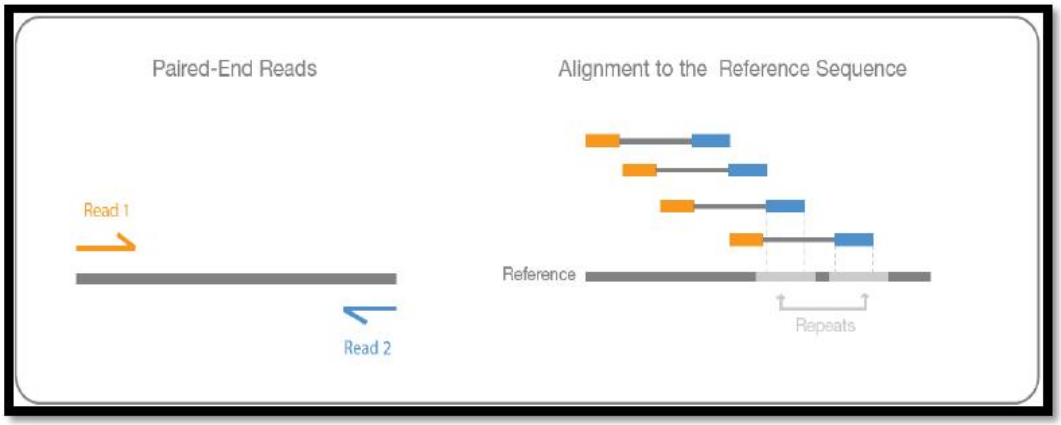


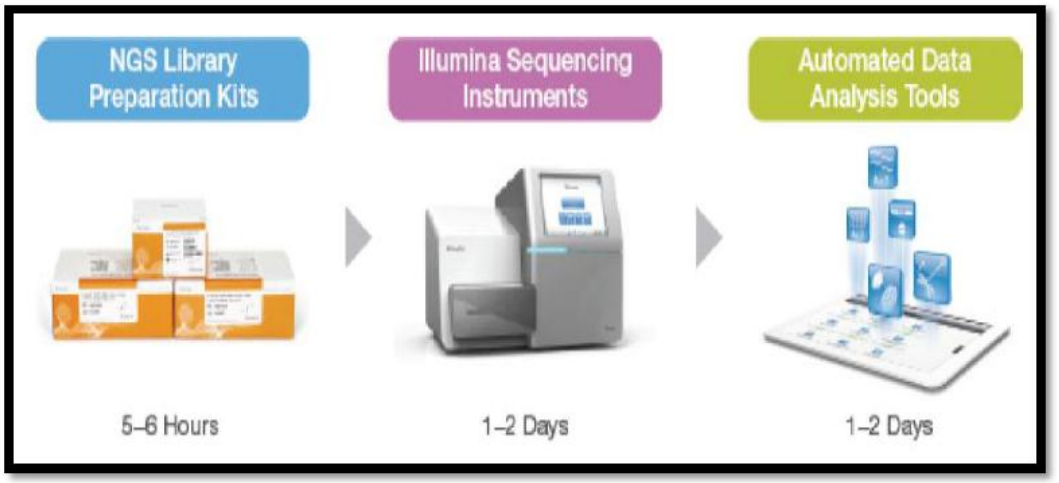
ILLUMINA SEQUENCING

FOUR BASIC STEPS IN ILLUMINA NGS WORK FLOW

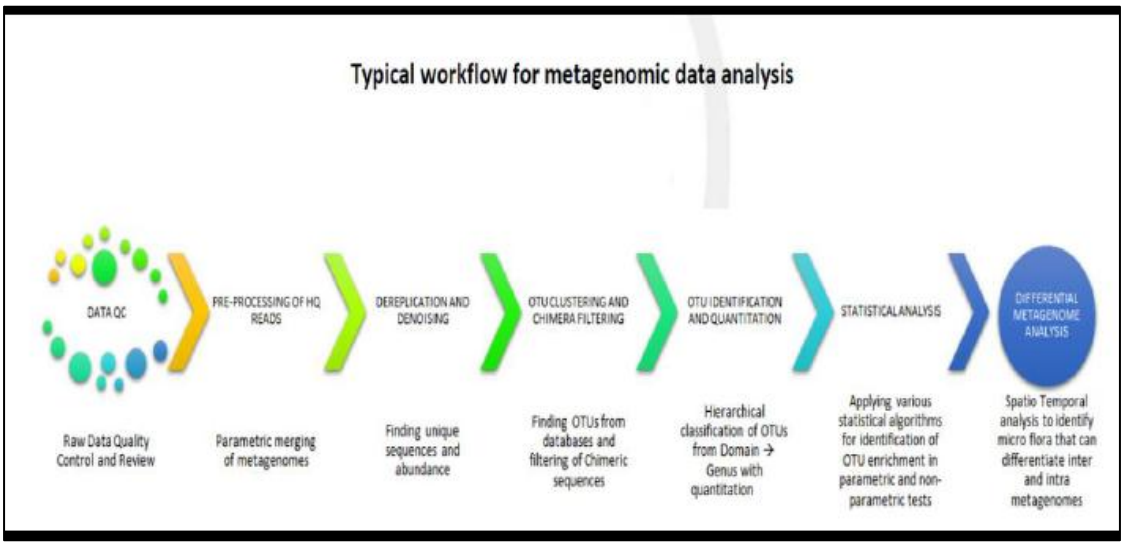


PAIRED- END SEQUENCING AND ALIGNMENT





WORKFLOW FOR METAGENOMIC DATA ANALYSIS



Results

RESULTS

The present study was carried out among 10 individuals in each group, that is in health and diseased individuals seeking dental treatment in Ragas Dental College and Hospital, Chennai. The age distribution of the study participants ranged from 20-53 years with a mean age of 32.66 years.

Saliva samples were collected in a saliva tub from periodontally healthy individuals (designated as H1,H2, H3,H4,H5,H6,H7,H8,H9 and H10) and from the periodontitis patients (designated as P1,P2,P3,P4,P5,P6,P7,P8,P9 and P10)

Orange complex bacteria included *Campylobacter rectus, Campylobacter gracilis, Campylobacter showae, Eubacterium nodatum, Fusobacterium nucleatum nucleatum, Fusobacterium nucleatum vincentii, Fusobacterium nucleatum polymorphum, Fusobacterium nucleatum periodonticum, Prevotella intermedia, Prevotella nigrescens, Peptostreptococcus micros* and *Streptococcus constellatus*.

TABLE 1: DISTRIBUTION OF ORANGE COMPLEX IN HEALTH GROUP

S.NO	ORANGE COMPLEX	H1	H2	H3	H4	H5	H6	H7	H8	H9	H10
1	Campylobacter showae	-	-	-	-	-	-	-	-	-	-
2	Campylobacter gracilis	-	**1**	**1**	-	-	**1**	-	-	-	-
3	Campylobacter rectus	-	**1**	-	-	-	**1**	-	-	-	-
4	Fusobacterium nucleatum nucleatum	-	-	-	-	-	-	-	-	-	-
5	Fusobacterium nucleatum polymorphum	**1**	**1**	**1**	-	**1**	**1**	**1**	-	-	**1**
6	Fusobacterium nucleatum Vincentii	**2**	**2**	**2**	**1**	**2**	**2**	**3**	**3**	**3**	**2**
7	Fusobacterium nucleatum Periodonticum	-	-	-	-	-	-	-	-	-	-
8	Eubacterium nodatum	-	-	-	-	-	-	-	-	-	-
9	Prevotella intermedia	-	-	-	-	-	-	-	-	-	-
10	Prevotella nigrescens	**1**	**1**	**1**	-	-	**1**	-	-	-	-
11	Peptostreptococcus micros	**1**	-	-	-	-	-	-	-	-	-
12	Streptococcus constellatus	-	-	-	-	-	-	-	-	-	-

Our results showed that in health samples *Fusobacterium nucleatum polymorphum* was present in 7 out of 10 samples (H1,H2,H3,H5,H6,H7,H10), *Prevotella nigrescens* was present in 4 samples (H1,H2,H3,H6), *Campylobacter gracilis* was present in 3 samples (H2, H3,H6), *campylobacter rectus* was present in 2 Samples (H2,H6), *Peptostreptococcus micros* was present in only 1 sample (H1), whereas *Fusobacterium nucleatum vincentii* was present in all the healthy individuals.

Campylobacter showae, Fusobacterium nucleatum nucleatum, Fusobacterium nucleatum periodonticum, Eubacterium nodatum, Prevotella intermedia and Streptococcus constellatus were not detected in any of the health samples examined in this study.

TABLE 2: DISTRIBUTION OF ORANGE COMPLEXES IN PERIODONTITIS GROUP

S.NO	ORANGE COMPLEX	P1	P2	P3	P4	P5	P6	P7	P8	P9	P10
1	Campylobacter showae	-	-	-	-	-	-	-	-	-	-
2	Campylobacter gracilis	**1**	**1**	**1**	**1**	**1**	**1**	**1**	**1**	**1**	**1**
3	Campylobacter rectus	**1**	-	**1**	**1**	**1**	**1**	**1**	**1**	**1**	**1**
4	Fusobacterium nucleatum nucleatum	**1**	-	**1**	-	**1**	-	-	-	**1**	**1**
5	Fusobacterium nucleatum polymorphum	**1**	**1**	-	-	**1**	**1**	**1**	-	**1**	-
6	Fusobacterium nucleatum Vincentii	**3**	**3**	**3**	**2**	**3**	**3**	**3**	**2**	**3**	**3**
7	Fusobacterium nucleatum Periodonticum	**1**	**1**	**1**	**1**	**1**	**1**	**1**	-	**1**	**1**
8	Eubacterium nodatum	-	-	-	-	-	-	-	-	-	-
9	Prevotella intermedia	**1**	**1**	-	**1**	**1**	-	-	-	**1**	**1**
10	Prevotella nigrescens	**1**	-	-	**1**	**1**	**1**	-	-	**1**	**1**
11	Peptostreptococcus micros	-	-	-	-	-	-	-	-	-	-
12	Streptococcus constellatus	-	-	-	-	-	-	-	**1**	**1**	-

The results showed that in Periodontitis samples *Campylobacter gracilis* and *Fusobacterium nucleatum vincentii* was present in all the 10 samples (P1,P2,P3,P4,P5,P6,P7,P8,P9,P10), was present in *Campylobacter rectus* was present in 9 (P1,P3,P4,P5,P6,P7,P8,P9,P10), *Fusobacterium nucleatum periodonticum* was present in 9 samples(P1,P2,P3,P4,P5,P6,P7,P9,P10), *Fusobacterium nucleatum polymorphum* was present in 6 samples (P1,P2,P5,P6,P7,P9), *Prevotella intermedia* was present in 6 samples (P1,P2,P4,P5,P9,P10), *Prevotella nigrescens* was present in 6 samples (P1,P4,P5,P6,P9,P10). *Fusobacterium nucleatum nucleatum* was present in 5 samples (P1,P3,P5,P9,P10) and *Streptococcus constellatus* was present in 2 samples (P8,P9).

Campylobacter showae, Eubacterium nodatum and Parvimonas micros were not detected in any of the periodontitis samples examined in this study.

Fusobacterium nucleatum vincentii was present in all the 20 samples examined including periodontal health and disease. On the other hand, *Eubacterium nodatum and campylobacter showae* were not present in any of the 20 samples examined.

Fusobacterium nucleatum nucleatum, Fusobacterium nucleatum periodonticum, Prevotella intermedia and Streptococcus constellatus were present in periodontitis but not in healthy samples. These results are represented in **Table -3**

TABLE- 3: ORANGE COMPLEX BACTERIA PRESENT IN PERIODONTITIS AND NOT IN HEALTH

Orange Complex	Expression	Healthy - n(%)	Periodontitis – n(%)	P value
C.gracilis	0	7 (70%)	1 (10%)	0.020*
	1	3 (30%)	9 (90%)	
F.N. nucleatum	0	10 (100%)	5 (50%)	0.033*
	1	-	5 (50%)	
F.N. periodonticum	0	10 (100%)	1 (10%)	0.000*
	1	-	9 (90%)	
P. intermedia	0	10 (100%)	4 (40%)	0.011*
	1	-	6 (60%)	
S. constellatus	0	10 (100%)	8 (80%)	0.474
	1	-	2 (20%)	

*Sig level at ≤0.05 (Mann whitney U test)

The results in **Table 3** suggest that salivary levels of *Campylobacter gracilis, Fusobacterium nucleatum nucleatum, Fusobacterium nucleatum periodonticum* and *Prevotella intermedia* were statistically significantly increased in periodontitis when compared to health. *Streptococcus constellatus* was present only in disease but there was no statistically significant difference when compared to health.

There was a statistically significant increase in frequency distribution of Campylobacter gracilis P=0.020 in periodontitis when compared to health.

Tables & Graphs

TABLE 1: DISTRIBUTION OF ORANGE COMPLEX IN HEALTH GROUP

S.NO	ORANGE COMPLEX	H1	H2	H3	H4	H5	H6	H7	H8	H9	H10
1	Campylobacter showae	-	-	-	-	-	-	-	-	-	-
2	Campylobacter gracilis	-	**1**	**1**	-	-	**1**	-	-	-	-
3	Campylobacter rectus	-	**1**	-	-	-	**1**	-	-	-	-
4	Fusobacterium nucleatum nucleatum	-	-	-	-	-	-	-	-	-	-
5	Fusobacterium nucleatum polymorphum	**1**	**1**	**1**	-	**1**	**1**	**1**	-	-	**1**
6	Fusobacterium nucleatum Vincentii	**2**	**2**	**2**	**1**	**2**	**2**	**3**	**3**	**3**	**2**
7	Fusobacterium nucleatum Periodonticum	-	-	-	-	-	-	-	-	-	-
8	Eubacterium nodatum	-	-	-	-	-	-	-	-	-	-
9	Prevotella intermedia	-	-	-	-	-	-	-	-	-	-
10	Prevotella nigrescens	**1**	**1**	**1**	-	-	**1**	-	-	-	-
11	Peptostreptococcus micros	**1**	-	-	-	-	-	-	-	-	-
12	Streptococcus constellatus	-	-	-	-	-	-	-	-	-	-

TABLE 2: DISTRIBUTION OF ORANGE COMPLEXES IN PERIODONTITIS GROUP

S.NO	ORANGE COMPLEX	P1	P2	P3	P4	P5	P6	P7	P8	P9	P10
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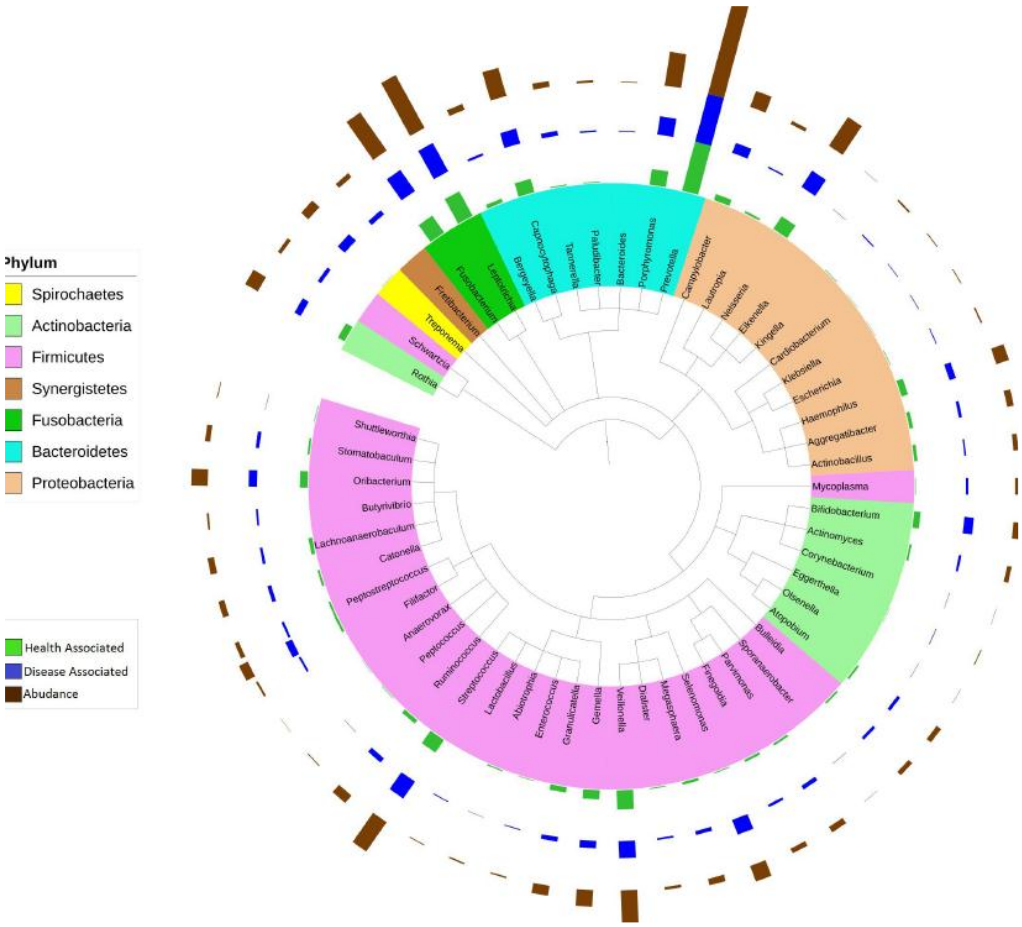
2	Campylobacter gracilis	**1**	**1**	**1**	**1**	**1**	**1**	**1**	**1**	**1**	**1**
3	Campylobacter rectus	**1**	-	**1**	**1**	**1**	**1**	**1**	**1**	**1**	**1**
4	Fusobacterium nucleatum nucleatum	**1**	-	**1**	-	**1**	-	-	-	**1**	**1**
5	Fusobacterium nucleatum polymorphum	**1**	**1**	-	-	**1**	**1**	**1**	-	**1**	-
6	Fusobacterium nucleatum Vincentii	**3**	**3**	**3**	**2**	**3**	**3**	**3**	**2**	**3**	**3**
7	Fusobacterium nucleatum Periodonticum	**1**	**1**	**1**	**1**	**1**	**1**	**1**	-	**1**	**1**
8	Eubacterium nodatum	-	-	-	-	-	-	-	-	-	-
9	Prevotella intermedia	**1**	**1**	-	**1**	**1**	-	-	-	**1**	**1**
10	Prevotella nigrescens	**1**	-	-	**1**	**1**	**1**	-	-	**1**	**1**
11	Peptostreptococcus micros	-	-	-	-	-	-	-	-	-	-
12	Streptococcus constellatus	-	-	-	-	-	-	-	**1**	**1**	-

TABLE- 3: ORANGE COMPLEX BACTERIA PRESENT IN PERIODONTITIS AND NOT IN HEALTH

Orange Complex	Expression	Healthy - n(%)	Periodontitis – n(%)	P value
C.gracilis	0	7 (70%)	1 (10%)	0.020*
	1	3 (30%)	9 (90%)	
F.N. nucleatum	0	10 (100%)	5 (50%)	0.033*
	1	-	5 (50%)	
F.N. periodonticum	0	10 (100%)	1 (10%)	0.000*
	1	-	9 (90%)	
P. intermedia	0	10 (100%)	4 (40%)	0.011*
	1	-	6 (60%)	
S. constellatus	0	10 (100%)	8 (80%)	0.474
	1	-	2 (20%)	

*Sig level at ≤0.05 (Mann whitney U test)

FIGURE: 8 PHYLOGENETIC TREE AT GENUS LEVEL



Discussion

DISCUSSION

Periodontal diseases are polymicrobial, multifactorial diseases with many host related factors that are involved in determining the individual susceptibility to disease. It is recognized that the relationship between periodontal microbiota and the host is generally benign but, changes in the subgingival microflora may cause periodontal inflammation and destruction with attachment loss and bone loss. [120,121,122] The role of subgingival microflora is sought to the explained by polymicrobial synergy and dysbiosis model proposed by **Hajishengalis**[38,39]

Human body comprises of various microbial colonies in which the microbes remain in the state of symbiosis to maintain the oral health and homeostasis. Various ecological niches are present within the oral cavity which may be distinct for buccal mucosa, tongue, palate, tonsils etc. **Turnbaugh et al**[132] classified human microbiome into two distinct types namely, core microbiome and variable microbiome. Core microbiome refers to the predominant species that exist under healthy conditions at different sites of the body, and it is shared by all individuals. Variable microbiome on the other hand has evolved in response to unique lifestyle, phenotypic and genotypic determinants and is exclusive to an individual.

Periodontal microflora may also be categorized into core and variable microbiome. Individual, regional and racial differences are known to

contribute to the variations observed in the subgingival microbiome across populations.

The Indian subgingival microbiome is yet to be fully characterized and there is sparse literature about the salivary microflora in our populations.

The **orange** complex is constituted by *Fusobacterium nucleatum, Prevotella intermedia, Prevotella nigrescens, Peptostreptococcus micros, Streptococcus constellatus, Eubacterium nodatum, Campylobacter showae, Campylobacter gracilis*, *Campylobacter rectus.*

In this study, orange complex has been evaluated in individuals in periodontal health and disease for the following reasons.

1. The species in this group are closely associated with one another and coaggregate with the red complex bacteria. Coaggregation has been shown to be a highly specific mechanism by which dental plaque bacteria may interact physically with other bacteria.[55] Most authors have described a potential role for coaggregation in formation of dental plaque biofilms and in particular secondary colonisation and development of a spatially organized community. [46] Coaggregation may provide some metabolic advantages to bacteria through cross-feeding, enzyme complementation and physical proximity.[25]
2. **Hajishengalis** et al[38,39] have also proposed that these bacteria may act as pathobionts and favour progression of the inflammatory process that is probably initiated by the red complex bacteria.

Salivary diagnostic aids are used as the biomarkers for prediction of periodontal disease activity because of their non-invasive ease of collection and bio availability of most molecules present in the GCF and serum. As most studies have focused on host related biomarkers, salivary profiling has largely been restricted to genomic, transcriptomic and proteomic analysis.[42] Salivary microbiome analysis and a potential profiling has received much less attention although it is widely accepted to be a medium for the translocation of oral bacteria.

Hitherto, culture based or Closed ended techniques like PCR have been largely relied upon to detect salivary microflora. In the current study, we have used the NGS- Next Generation Sequencing technology for high-throughput genomic analysis.

NGS technology is known to be a high-throughput genomic analysis technique and is an open-ended diagnostic approach. This methodology is in accordance with previous studies of **Griffen et al[32], Hong et al, Kumar et al[61],** who have used NGS to characterize the subgingival microbiome.

The advantages of this method include that the entire bacterial species present in the subgingival environment are simultaneously identified and quantified.

1) Culture based methods cannot identify species whose culture characteristics are unknown. It has been estimated that there are nearly 300 and more uncultivable species are present in subgingival plaque.
2) Extremely sensitive closed ended techniques like DNA probes, RT-PCR can identify only targeted organisms against which specific primers have been designed.

Among the NGS technologies, Illumina sequencing has been used in this study for the following reasons,

1) It provides more sequence per run as a result of which there is a greater in-depth coverage than other technologies. This in turn helps to analyse a larger sample size, include more bar-coded time points and assess the total diversity in microbiome.
2) The low abundance taxa can be determined with generation and sequencing of short 16S rRNA amplicons.

However, the illumina sequencing remains almost prohibitively expensive for routine clinical and research use. We have therefore combined resources to use this technology and achieve a reasonable sample size. While other studies have focused on the red complex, yellow and green complexes, this study has evaluated the orange complex bacteria.

In this study, subjects were periodontally evaluated and allocated into two groups as healthy controls (ten subjects) and periodontitis patients (ten subjects). The periodontitis patients were selected and the sample was

collected in a sterile salivary tub using the method described by Navazesh. Unstimulated whole saliva was collected in the morning and subjects were advised to refrain from eating, drinking, smoking or performing any oral hygiene for at least 2 hours prior to the collection. The samples obtained were frozen and stored at -80°C until the sample collection period was completed.

In the experimental workflow, all reactions were carried out with water and plastic materials guaranteed as DNA-free to avoid. All the DNA extraction to analysis procedures was carried out by strictly adhering to manufacturer protocols and existing literature.

The salivary microbiome as a whole is represented at the genus level using the phylogenetic tree. **(FIGURE-8)**

The results of our study showed that *Fusobacterium nucleatum vincentii* was present in all the 20 samples examined including periodontal health and disease. These results are in accordance with previous studies that have shown that the phyla Fusobacteria was ubiquitously present in plaque and saliva.[1]

On the other hand, *Eubacterium nodatum and Campylobacter showae* were not present in any of the 20 samples examined. While the reason for this is not immediately apparent, these results are in accordance with the previous studies that have reported wide variations in subgingival microflora among populations. [1]

There was a statistically significant increase in salivary *Campylobacter gracilis, Fusobacterium nucleatum nucleatum, Fusobacterium nucleatum periodonticum, Prevotella intermedia. Streptococcus constellatus* was not statistically significant in periodontitis when compared to health because of the lesser number of samples in which it was present (n=2). **(TABLE -3)**

In comparison with results from earlier studies of **Liu et al and Wang et al[71],** our study reports a higher prevalence of Proteobacteria, Fusobacteria, Firmicutes and Bacteriodetes in periodontitis samples. Proteobacteria phylum comprises of species such as Campylobacter, Hemophilus, Mannheimia and Desulfobulbus; Whereas, Fusobacteria phylum comprises genera Fusobacterium and Leptotriciae; Bacteroidetes phyla consists of many of the organisms that have been associated with periodontal disease, such as, Porphyromonas, Tannerella, Capnocytophaga, Prevotella etc; all of which are known to be early colonizers.

In our study *Campylobacter gracilis* (P=0.020) of Proteobacteria phylum was observed to be present in all the periodontitis samples. It is one of the largest bacterial phyla and contains most of the Gram-negative bacteria known to be periopathogens such as *Actinobacter* and *Hemophilus* (gamma division), *Eikenella corrodens* (beta division) and *Campylobacter* (epsilon division).

Our results are in line with those of **P.J. MACUCHI**[77] who have shown that *Campylobacter gracilis* was found in initial periodontitis sites. The association of campylobacter species in the initial lesion was also demonstrated by various cultivable methods by **Tanner et al**[125]**.**

Other studies have also demonstrated that *Campylobacter gracilis* were elevated in moderate to advanced diseased human subgingival sites compared with non-diseased sites, suggesting that these species are directly associated with periodontal disease.[125,88,27]

Our results are however not in accordance with **Loreto** and **Amanda**[1], who suggested that *Campylobacter gracilis* species was one among the various species that form the core microbiome. This bacterium was present only in periodontitis and not in all the samples that were evaluated in this study.

In our study, *Fusobacterium nucleatum nucleatum (P=0.033) and Fusobacterium nucleatum periodonticum (P=0.000)* of phylum fusobacteria was present only in periodontitis patients. This was in line with earlier studies done by **Liu B et al**[71]**, Griffen AL et al**[32]**, Shi B et al**[117]. It is a well-established fact that Fusobacterium species play a major role as bridging organism in establishing periodontitis and possibly by making the environment conducive for oxygen intolerant anaerobes.[116]

Fusobacterium nucleatum is important for co-aggregation between early and late colonizers, thereby helping in subgingival biofilm organization;

this organism may be used as marker for transition from gingivitis to periodontitis and for further disease progression. Complexes formed by *F. nucleatum, B. forsythus* and *C. rectus* were also found in sites refractory to treatment. [11]

Other studies have demonstrated that Fusobacterium species were closely associated with one another and this complex appeared closely related to the red complex.[120,121,122]

Our results showed *Prevotella intermedia*, a gram-negative bacterium that belongs to the phylum Bacteriodetes was present only in periodontitis samples. Previous studies have shown that colonization by *P. intermedia* was found to be due to *F. nucleatum*, since *P. intermedia* was never detected in a site unless *F. nucleatum* was also present.[11]

Other studies have reported that *Prevotella intermedia*, has been implicated as a putative periodontal pathogen due to its isolation from lesions of patients with early periodontitis, advanced periodontitis, and acute necrotizing ulcerative gingivitis.[120,121,122]

Our results showed *Streptococcus constellatus P=0.474* which belonged to the phyla Firmicutes was observed only in periodontitis patients. However, there was no statistically significant difference from the health samples as it was isolated only from two of the ten periodontitis samples. Therefore, its role in etiopathogenesis and as a biomarker is uncertain.

There is some evidence in literature to suggest that *S. constellatus* colonises with *S. intermedius*. Their strong anti-phagocytic resistance to human polymorphonuclear leukocytes may contribute to their association with

forms of periodontal disease that do not respond to conventional mechanotherapy.[78,92]

Other studies have reported that *Streptococcus constellatus* was often found with *Eikenalla corrodens,* on observation that the *Eikenella* species stimulates the growth of *S. constellatus.*[144]

It must be remembered that most literature relating to microbial etiology and pathogenesis of periodontal disease is related to the plaque environment. Our results are therefore to be interpreted with some caution when applied to etiopathogenesis of periodontal disease. Co-aggregation and other virulence mechanisms may differ in subgingival plaque environment when compared to saliva.

Recent literature however suggested that there may be migrating microbiomes which may play a role in dissemination and subsequent pathology.[16] In the light of scanty information available to us, presently their role is yet to be fully understood.

A relatively sample size and the cross-sectional nature are some of the limitation associated with this study.

Within the limits of the study it appears that salivary *Campylobacter gracilis,* Fusobacterium *nucleatum nucleatum, Fusobacterium nucleatum periodonticum, Prevotella intermedia and Streptococcus constellatus levels* may be used as the markers of periodontal disease. Further long term studies with a greater sample size are required to substantiate these results.

Summary & Conclusion

SUMMARY AND CONCLUSION

This study characterized the orange complex in the microbiome of saliva in periodontally healthy and periodontitis individuals. Twenty saliva samples were taken including ten periodontally healthy and ten periodontitis samples were collected and salivary microbiome characterization was done with NGS technology using Illumina sequencing.

Fusobacterium nucleatum vincentii was present in all the 20 samples examined including periodontal health and disease. On the other hand, *Eubacterium nodatum and Campylobacter showae* were not present in any of the 20 samples examined.

Among the orange complex bacteria *Campylobacter gracilis (P=0.020), Fusobacterium nucleatum nucleatum (P=0.033), Fusobacterium nucleatum periodonticum (P=0.000), Prevotella intermedia (P=0.01)* were statistically significant in periodontitis when compared to health; Whereas *Streptococcus constellatus (P=0.474)* was present only in periodontitis but there were no statistically significant differences with health.

Therefore, they may be considered as the microbial risk markers. Especially *Campylobacter gracilis (p=0.020)* as it is present in all the diseased samples. Further long-term studies with a higher sample size need to be done to confirm these findings.

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Annexures

ANNEXURE- I

RAGAS DENTAL COLLEGE & HOSPITAL
(Unit of Ragas Educational Society)
Recognized by the Dental Council of India, New Delhi
Affiliated to The Tamilnadu Dr. M.G.R. Medical University, Chennai
2/102, East Coast Road, Uthandi, Chennai - 600 119. INDIA.
Tele : (044) 24530002, 24530003 - 06. Principal (Dir) 24530001 Fax : (044) 24530009

TO WHOMSOEVER IT MAY CONCERN

Date: 30.1.2019

Place: Chennai.

From

The Institutional Review Board,

Ragas Dental College and Hospital,

Uthandi, Chennai- 600119.

The Dissertation topic titled **"ANALYSIS OF ORANGE COMPLEX BACTERIA IN THE MICROBIOME OF WHOLE SALIVA IN PERIODONTAL HEALTH AND PERIODONTITIS INDIVIDUALS USING NEXT GENERATION SEQUENCING TECHNOLOGY"** Submitted by **DR. ENNET CYNTHIA JOHNS** has been approved by the Institutional Review Board of Ragas Dental College & Hospital.

Dr.N.S. AZHAGARASAN, M.D.S.
Member Secretary,
Institutional Ethical Board,
Ragas Dental College and Hospital,
Uthandi, Chennai – 600119.

ANNEXURE- II

URKUND

Urkund Analysis Result

Analysed Document:	ENNET CYNTHIA JOHNS 1.docx (D47312844)
Submitted:	1/28/2019 6:04:00 AM
Submitted By:	drcynthiajohns@gmail.com
Significance:	5 %

Sources included in the report:

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Instances where selected sources appear:

23

ANNEXURE – III

CONSENT FORM

I ……S/o, w/o, d/o……

aged about ……years, Hindu/Christian/Muslim …… ……residing at ……do solemnly And state as follows.

I am the deponent herein; as such I am aware of the facts stated here under

I state that I came to Ragas Dental College and Hospital, Chennai for my treatment for

……

……

I was examined by Dr…….. and I was requested to do the following

1. Full mouth Plaque Score
2. Full mouth bleeding score

3 Measurement of periodontal pocket depth and clinical attachment loss

I was also informed and explained about the collection of plaque during scaling in ……(language) known to me.

I was also informed and explained that the results of the individual test will not be revealed to the public. I give my consent after knowing full consequence of the

dissertation/thesis/study and I undertake to cooperate with the doctor for the study.

I also authorise the Doctor to proceed with further treatment or any other suitable alternative method for the study,

I have given voluntary consent to the collection of plaque for approved research.

I am also aware that I am free to withdraw the consent given at any time during the study in writing.

Signature of the patient/Attendant

The patient was explained the procedure by me and has understood the same and with full consent signed in (English/Tamil/Hindi/Telugu?.....) before me

Signature of the Doctor